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*CONTRASTS IN THE METABOLIC STABILITY OF DIFFERENT NUCLEOTIDES IN THE RIBONUCLEIC ACIDS OF ISOLATED NUCLEI**

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THE ROCKEFELLER INSTITUTE

Communicated July 27, 1959

The present communication is concerned with ribonucleic acid synthesis in the cell nucleus and with some striking differences in the metabolic stability of different nucleotides following their incorporation into the RNA molecule. The experiments to be described indicate that the uridine-containing nucleotides of nuclear RNA are labile, and that they take part in a rapid "turnover" reaction, the rate of which increases when uridine is added to the suspension medium. On the other hand, there is no evidence for a rapid "turnover" of either the cytidylic or adenylic acid components of nuclear RNA.

This intramolecular "turnover" of the uridine containing nucleotides was detected in the course of experiments on RNA synthesis in isolated thymus nuclei. In such experiments the RNA's of the nucleus are labeled by incubation *in vitro* with different radioactive precursors. We have previously shown that isolated nuclei, under the proper conditions, can incorporate orotic acid-6-C^{14} into the pyrimidines, and glycine-1-C^{14} into the purines of their ribonucleic acids.[1, 2] This activity, and the corresponding uptake of adenosine-8-C^{14} into RNA, were found to require the presence of the deoxyribonucleic acids of the cell nucleus.[1, 3] This DNA-dependence was also observed in parallel studies of amino acid uptake into nuclear proteins.[1, 3] It was then shown that RNA and protein synthesis are both energy-dependent, and that DNA plays a role in mediating the synthesis of ATP by the nucleus.[4]

Because the uptake of radioactive amino acids into proteins need not necessarily represent protein synthesis, we regularly tested to see whether C^{14}-amino acids, once incorporated into nuclear proteins, would exchange with C^{12}-amino acids added to the incubation medium.[1] The test involved labeling the nuclear proteins by a short incubation with a C^{14}-amino acid, washing away the isotope, and then reincubating the nuclei in the presence of a large excess of the corresponding C^{12}-amino acid. The protein was isolated at different times and its specific radioactivity was measured. A similar technique was used successfully by Gale and Folkes[5] and by Nisman *et al.*[6] in demonstrating amino acid "exchange" reactions in bacterial systems. It was also applied by Harris and Watts[7] in a study of valine "turnover" in the proteins of rabbit macrophages. In thymocyte nuclei, however,

there is no evidence for a rapid loss or "exchange" of amino acids following their incorporation into protein.

Similar tests for isotope retention have now been applied to nuclear RNA's, using four different radioactive precursors, and varied conditions of incubation.

The results indicate that the metabolism of ribonucleic acids in the cell nucleus is more complex than had been surmised, and that the pattern of incorporation and retention of different precursors can be used to shed light on some interesting synthetic pathways and contrasts in the metabolic stability of different portions of the RNA molecule.

Incorporation Processes in Isolated Nuclei.—The uptake and retention of adenosine-8-C^{14}: The preparation of isolated nuclei from homogenates of calf thymus tissue has been described previously.[8, 1] The isolation medium is 0.25 *M* sucrose-0.003 *M* $CaCl_2$, an isotonic solution which has been found to maintain many of the synthetic capacities of the thymocyte nucleus. The purity of these nuclear fractions has been tested under the electron microscope, and in other ways.[8, 9] Electron microscopy shows 29–77 cells per thousand nuclei, the higher figure including not only intact cells, but also nuclei with tabs of adhering cytoplasm. The lower figure for whole cell contamination is in accord with the results of Ficq and Errera[10] who examined the nuclear fraction after staining with Unna's stain, and found 3 per cent intact cells. Both light and electron microscopy support the results of chemical and enzymatic tests which show the nuclear fractions to be better than 90 per cent pure.

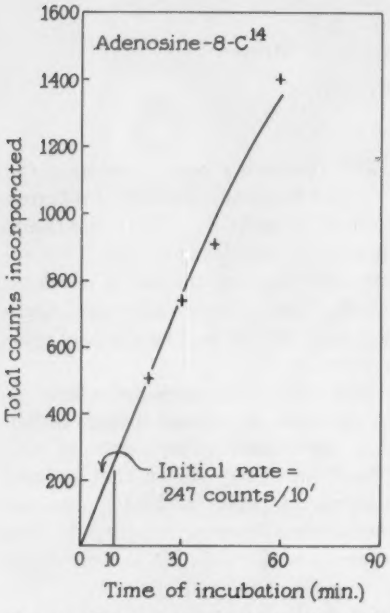

FIG. 1.—The time course of adenosine-8-C^{14} incorporation into the RNA of isolated thymus nuclei. The total radioactivity of the ribonucleic acid is plotted against the time of incubation. Each flask contained 1.0 ml of nuclear suspension (containing 40 mg nuclei), 0.5 ml 0.1 *M* sodium phosphate buffer in 0.25 *M* sucrose (pH 6.7), 0.4 ml 0.1 *M* glucose containing 3.75 mg NaCl + 4.19 mg $MgCl_2 \cdot 4H_2O$ per ml, and 0.1 ml H_2O containing 100 μg (67,500 counts) of adenosine-8-C^{14}.

Isolated thymus nuclei are active in many incorporation systems, including amino acid uptake into protein,[1] thymidine uptake into DNA,[11] and adenosine and orotic acid uptake into RNA.[1, 2] The work now to be described deals with the comparative incorporation and retention of four different RNA precursors; adenosine-8-C^{14}, orotic acid-6-C^{14}, totally labeled uridine-C^{14}, and P^{32}-orthophosphate.

The time course of adenosine incorporation into nuclear RNA is shown in Figure 1. The ordinate gives the total counts incorporated into the RNA of 40 mg of thymus nuclei (dry weight) following incubation for different times in the presence of 100 μg (67,500 counts) of adenosine-8-C^{14}. (The incubation conditions are given

in detail in the legend to the figure.) That the uptake of labeled adenosine actually represents incorporation into RNA has been shown in several ways. First, alkaline hydrolysis (and subsequent acidification to precipitate the DNA) releases 94 per cent of the counts as RNA mononucleotides. Similarly, treatment of the nuclei with ribonuclease in a neutral buffer releases 89 per cent of the radioactivity incorporated. (As usual, this enzymatic test for localization in RNA is complicated by the fact that the neutral buffer can itself extract some RNA from the nucleus. However, all the RNAase extracts show the UV absorption spectra and color reactions (i.e., a positive orcinol test, and a negative diphenylamine reaction) expected for ribonucleotides.) A third test was the isolation of RNA by a modification of Kirby's phenol procedure[12] and the measurement of its C^{14}-activity. All three tests agree in showing the localization of isotope in material with the properties of ribonucleic acids. (More specific tests for the localization of C^{14} derived from orotic acid and uridine, and of P^{32} from orthophosphate will be described subsequently.)

The initial slope of the adenosine incorporation curve (25 cpm per minute) corresponds to the uptake of 2.22 μg of adenosine-8-C^{14} per hour. From the amount of isotopic adenosine incorporated an estimate may be made of the amount of new RNA which may be formed. The RNA of the nucleus is 16 per cent adenosine by weight. If all the uptake under these conditions is considered to represent RNA *synthesis* (and experiments described below suggest that this is largely the case), it can be calculated that 13.8 μg of RNA are synthesized per hour. Although this is a small increase relative to the weight of nuclei present (40 mg or 1.3×10^9 nuclei) the extent of the synthesis can be better appreciated from the calculation that in this interval the average nucleus is making over 3000 molecules of RNA (of molecular weight 2 million).

The uptake of adenosine by the nucleus is, in part, sodium dependent. In this respect it resembles amino acid incorporation into nuclear protein.[1] Other indications of a relationship between RNA and protein synthesis in the nucleus were observed previously, when it was found that certain benzimidazole derivatives prevent RNA synthesis. A sequel to this effect is the inhibition of amino acid uptake into protein.[1] More direct evidence for the role of RNA in nuclear protein synthesis has been obtained recently.[13] Some nuclear RNA's have been shown to function as "carriers" of amino acids following their activation by reaction with ATP. The role of ions in these early stages of protein synthesis is now under investigation. At present it appears that there is no specific sodium ion requirement for ATP synthesis or for amino acid activation.[13] It remains to be seen whether the common ionic requirement for adenosine uptake and amino acid incorporation indicates any subsequent liaison between RNA and protein synthesis.

Evidence will now be presented to show that the incorporation of adenosine-C^{14} into nuclear RNA is largely, but not entirely, irreversible. The test for the stability of adenosine in RNA was carried out in the following way. Nuclei were incubated with 100 μg of adenosine-8-C^{14} in the usual manner. After 30 minutes the nuclei were centrifuged down, and the supernatant fluid (which contains over 80 per cent of the labeled adenosine originally present) was discarded. The nuclei were then washed by suspending them in 7 volumes of incubation medium containing 1000 μg of unlabeled (C^{12}) adenosine per ml. The nuclei were centrifuged down and washed again in the same way (each of these washings contained about a 350-fold excess of

unlabeled adenosine). Following centrifugation, the nuclei were resuspended in C^{14}-free incubation medium, and aliquots of the suspension were shaken at 38°C for periods up to 180 minutes. The total counts present in nuclear RNA was then determined as previously described,[2] and plotted against the time of incubation. For purposes of comparison it is convenient to express the radioactivity present at different times as a per cent of the counts present at the outset of the incubation (t = 15 minutes). The results are shown in Figure 2. The upper curve summa-

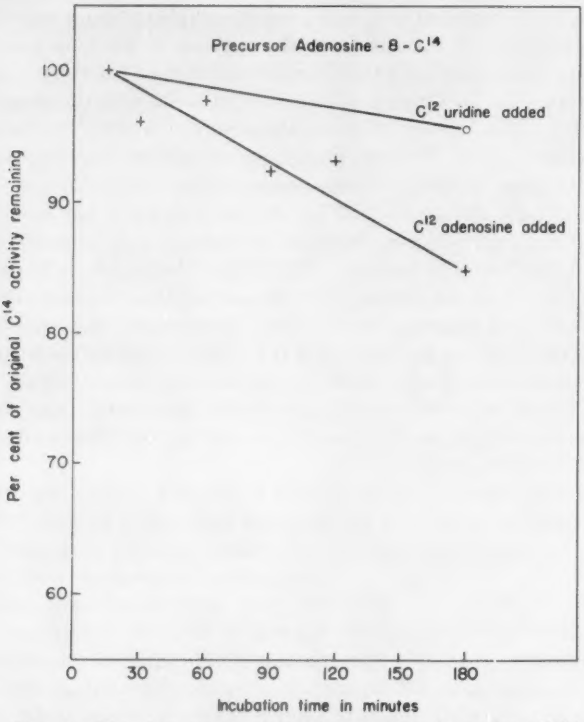

FIG. 2.—The time course of isotope retention in nuclear RNA previously labeled with adenosine-8-C^{14}. The total radioactivity present in the nuclear RNA at different times is expressed relative to the C^{14} activity at t = 15 minutes. The medium is that described in the legend to Fig. 1 (except that no labeled adenosine was present during the retention experiments). For further details see text.

rizes the experiments carried out in the absence of added C^{12}-adenosine; the lower curve shows the extent of isotope retention in the presence of 0.005 M adenosine.

It is clear that the addition of unlabeled adenosine to the medium increases the rate of loss of C^{14}-adenosine previously incorporated into RNA. The addition of uridine (0.005 M) has no such effect on adenosine "turnover," and the C^{14} loss follows the upper "control" curve. The uridine supplement was tested because C^{12}-uridine markedly accelerates the rate of loss of C^{14} from the uridylic acid of nuclear RNA (see below).

In the absence of added C^{12}-adenosine, the loss of isotope from adenosine-C^{14} labeled RNA is a small one; only 4 per cent of the counts are lost in the 3-hour incubation period. Even with C^{12}-adenosine present, the C^{14} label is relatively stable, and nearly 85 per cent of the counts remain after 3 hours. This contrasts with the results to be described below, which indicate a far greater lability of the uridylic acid of nuclear RNA.

The Incorporation of Orotic-6-C^{14} Acid into Nuclear RNA and Its Subsequent Metabolism.—The uptake of C^{14}-orotic acid by isolated nuclei has been described previously.[1, 2] At that time it was pointed out that nuclear ribonucleic acid is metabolically heterogeneous, and that the RNA of the nucleolar fraction is far more active than the RNA fraction which can be extracted in neutral salt solutions.[2] The experiments now to be described show: (*1*) *that isolated nuclei utilize orotic acid for the synthesis of both the uridylic and cytidylic acids of RNA*, (*2*) *that the cytidylic acid incorporated into RNA is metabolically stable*, and (*3*) *that the uridylic acid in nuclear RNA takes part in a "turnover" reaction which is indicated by a loss of C^{14} activity.* This loss is accelerated by adding unlabeled uridine to the medium.

TABLE 1

INCORPORATION OF OROTIC ACID-6-C^{14} AND URIDINE C^{14} INTO THE NUCLEOTIDES OF NUCLEAR RIBONUCLEIC ACID

Experiment	C^{14}-Precursor	Incubation Conditions	Specific Activity of Cytidylic Acid	Specific Activity of Uridylic Acid
1	Orotic acid-6-C^{14}	*a*	1,250	19,500
			1,210	19,800
2	Uridine-C^{14}	*b*	224	1,220
3	Orotic acid-6-C^{14}	*a*	1,210	13,400
	Uridine-C^{14}	*b*	265	1,870
4	Orotic acid-6-C^{14}	*c*	1,230	8,310

Incubation conditions:
(*a*) 10.0 ml nuclear suspension, 5.0 ml 0.05 *M* potassium phosphate-0.125 *M* sucrose (pH 6.7), 4.0 ml 0.1 *M* glucose with 4 mg NaCl + 4.19 mg $MgCl_2 \cdot 4H_2O$/ml, 1.0 ml H_2O containing (40 μg = 0.44 μc) orotic acid-6-C^{14}.
(*b*) As above, containing 40 μg = 24,700 counts of randomly labeled uridine-C^{14} in place of C^{14} orotic acid.
(*c*) 10.0 ml nuclear suspension, 5.0 ml 0.1 *M* sodium phosphate-0.25 *M* sucrose (pH 6.7), 4.0 ml 0.1 *M* glucose with 3.75 mg NaCl + 4.19 mg $MgCl_2 \cdot 4H_2O$/ml, 1.0 ml H_2O containing (40 μg = 0.44 μc) orotic acid-6-C^{14}.
All shaken at 38° for 30 minutes.

The time course of orotic acid incorporation (presented previously[2]) resembles that of adenosine as shown in Figure 1. In other respects, however, the uptake of orotic acid-6-C^{14} differs markedly from that of other RNA precursors tested. One of the most striking differences is the absence of a specific sodium ion requirement for orotic acid incorporation. All of the sodium in the medium can be replaced by an equivalent amount of potassium without diminishing the extent of C^{14}-uptake. Another characteristic of orotic acid incorporation is that it proceeds best in a hypotonic medium. This can be shown by starting with nuclei in a very dilute incubation medium (0.156 *M* sucrose, 0.0015 *M* $CaCl_2$, 0.02 *M* glucose, 0.0125 *M* potassium phosphate buffer of pH 6.7) and adding increasing amounts of sodium chloride. Tests of C^{14} incorporation at the different salt concentrations show optimum uptake of orotic acid at 0.014 *M*, of C^{14}-uridine at 0.027 *M*, and of adenosine-8-C^{14} at 0.041 *M*. The optimum for amino acid incorporation is the same as that for adenosine.

The localization of the orotic acid label in nuclear RNA has been demonstrated in several ways. Digestion with ribonuclease releases 95 per cent of the radioactivity incorporated. Alkaline hydrolysis is even more effective, releasing 97 per

cent of the counts as RNA mononucleotides. The nucleotides can then be separated on Dowex-1 (formate) columns by a modification of the method of Hurlbert *et al.*[14, 15] Table 1 shows the distribution of radioactivity in the cytidylic acid and uridylic acid fractions. The specific activity of the cytidylic acid is low compared with that of uridylic acid, but *the results show definitely that the nucleus does utilize orotic acid for the synthesis of cytidylic acid in RNA*. In this respect it differs from cytoplasmic systems previously studied, in which orotic acid is used exclusively for uridylic acid synthesis.[16]

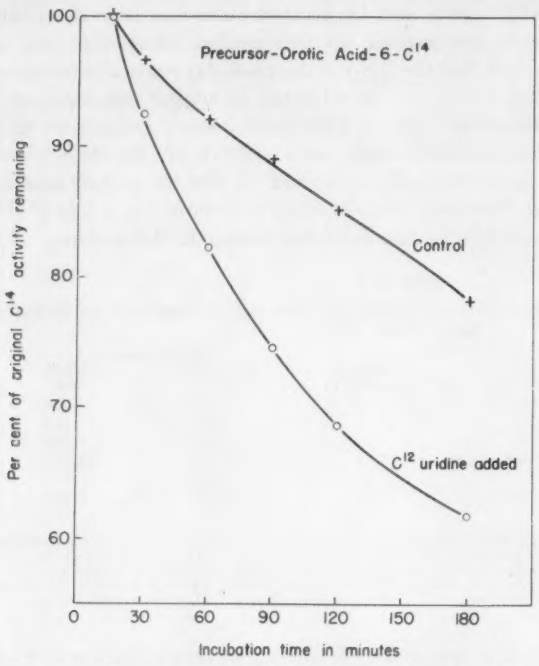

FIG. 3.—The time course of isotope loss from nuclear RNA previously labeled with orotic acid-6-C^{14}. The total radioactivity present in the nuclear RNA at different times is expressed relative to the C^{14} content at $t = 15$ minutes. The nuclei were labeled as described in Table 1, footnote [a]. The upper curve shows the loss of radioactivity in a medium containing 1.0 ml of nuclear suspension, 0.5 ml 0.05 *M* potassium phosphate buffer in 0.125 *M* sucrose (pH 6.7), 0.4 ml 0.1 *M* glucose containing 4 mg NaCl + 4.19 mg $MgCl_2 \cdot 4H_2O$ per ml, and 0.1 ml H_2O. The lower curve shows the loss of radioactivity when unlabeled uridine (0.005 *M*) is added to this medium.

The following experiments were carried out to test whether the incorporation of C^{14}-orotic acid into the uridylic and cytidylic acids of RNA is an irreversible process. The nuclear RNA was labeled by a 30-minute incubation in the presence of 40 μg (0.44 μc) of orotic acid-6-C^{14}. (The incubation medium is described in the legend for Figure 3.) The nuclei were centrifuged down and the supernatant was discarded. The sediment was then washed by resuspending it in seven volumes of incubation medium containing 1 mg of unlabeled orotic acid per ml. Following centrifugation, the nuclei were washed again in the same way. They were finally taken up in incubation medium, and aliquots of the suspension were shaken at 38° for periods of 15 to 180 minutes. The total radioactivity present in the nuclear RNA at different times was determined as previously described[2] and compared with that originally present at the outset of the incubation. The plot of isotope retention as a function of the time of incubation is given in Figure 3. The upper curve summarizes the results of 11 "control" experiments of this kind. The lower curve shows the rate of isotope loss when C^{12}-uridine (0.005 *M*) is present in the medium.

The "control" curve shows that about 22 per cent of the total C^{14} activity is lost

from the nuclei in a 3-hour period. The rate of C^{14} loss is nearly doubled when C^{12}-uridine is added to the medium, and 40 per cent of the counts are lost in 3 hours. It can be shown that *this loss of radioactivity from the nucleus is not due simply to a loss of nuclear RNA*. Ribonucleic acid analyses show that the RNA content of the nuclei at the end of the incubation is only 2–4 per cent lower than the amount originally present. (The Webb method of RNA analysis[17] was used instead of the usual orcinol color reaction because of its greater accuracy in the presence of large amounts of DNA.) In a typical experiment, the initial RNA content was 1.45 per cent of the dry weight of the nucleus; after 180 minutes' incubation, the value was 1.41 per cent. Moreover, the small amount of RNA which does leave the nucleus can be precipitated from the incubation medium by adding acid (final concentration 2% $HClO_4$). Measurements of its radioactivity show that it resembles the RNA which remains in the nucleus with respect to the progressive loss of C^{14} label. Other experiments (to be described below) show that the uridylic acid of the RNA remaining in the nucleus loses its radioactivity while the C^{14}-activity of the cytidylic (and adenylic) acids remains essentially constant.

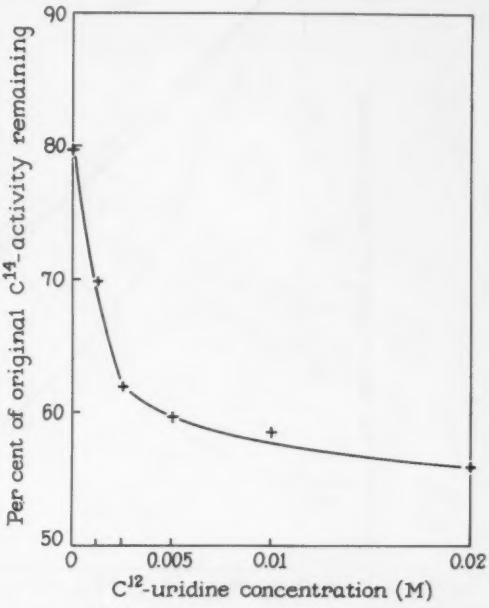

FIG. 4.—The effect of increasing C^{12}-uridine concentration on the loss of C^{14} from nuclear RNA previously labeled with orotic acid-6-C^{14}. The total radioactivity remaining after 3-hours' incubation when nuclei are suspended in different concentrations of unlabeled uridine is expressed relative to the C^{14} activity at t = 15 minutes. The medium is given in the legend to Fig. 3.

The retention experiments summarized in Figure 3 gave the first indication of a "turnover" of the uridylic acid in nuclear RNA. It is of interest that the rate of C^{14} loss increases when the nucleoside, uridine, is added to the medium. The effect of increasing uridine concentrations on the loss of C^{14} from nuclear RNA is shown in Figure 4. In these experiments the nuclei were incubated with orotic acid-6-C^{14} in the usual way, washed free of labeled precursor, and resuspended in the incubation medium containing the indicated concentrations of "cold" uridine. After 3 hours' incubation, the total radioactivity remaining in the nuclear RNA was compared with that present at the outset of the incubation. The amount of isotope retained is plotted against the uridine concentration of the suspending medium. All of the uridine experiments described in this paper were carried out at 0.005 *M;* at this concentration about 40 per cent of the C^{14} activity is lost in 3 hours.

This effect of uridine in promoting the loss of uridylic acid-C^{14} from nuclear RNA is relatively specific. The corresponding nucleotide, uridine-5′-phosphate (UMP) has no such effect; nor does uridine diphosphate (UDP). This is shown by the experiments summarized in Figure 5, which compares the rate of C^{14} loss in the presence of uridine (0.005 *M*) with that observed at equivalent concentrations of UMP or UDP. It is clear that the latter compounds do not influence the rate of C^{14} loss from the nucleus. Other tests have shown that a mixture of the 2′-and 3′-phosphates of uridine also fails to promote the loss of C^{14} from RNA.

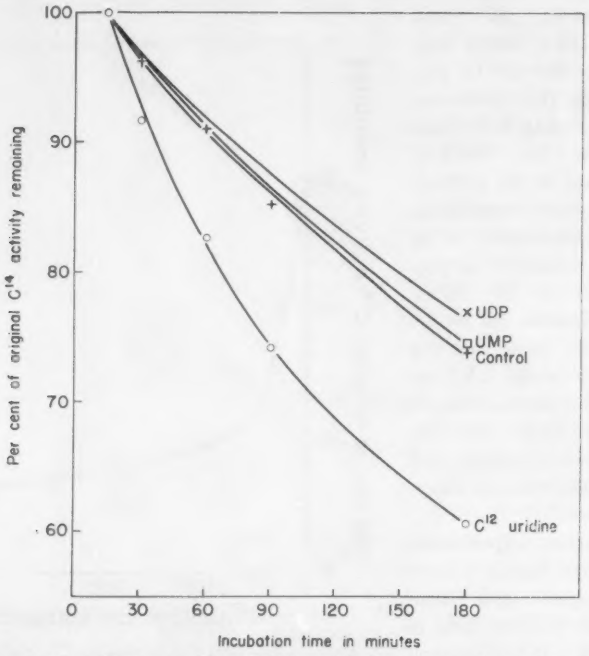

FIG. 5.—Comparative effects of uridine, UMP and UDP in promoting C^{14} loss from nuclear RNA previously labeled with orotic acid-6-C^{14}. The total activity remaining at different times is expressed relative to C^{14} activity at t = 15 minutes. The "control" medium is described in the legend to Fig. 3. Uridine, UMP, or UDP were added to this medium to give a final concentration of 0.005 *M*.

The failure of the phosphorylated nucleosides to affect the "turnover" of isotopically labeled RNA raises the question of whether these compounds can penetrate the isolated nucleus. This was tested by comparing the effects of UMP, UDP, and uridine on the uptake process. The incorporation of labeled orotic acid into RNA involves the prior formation of UMP (which can then be phosphorylated to UDP or UTP). If the nucleus can utilize unlabeled UMP or UDP added in large excess to the incubation medium, then one would expect the incorporation of labeled orotic acid to be proportionately decreased. This is found to be the case; measurements of orotic acid-6-C^{14} uptake into nuclear RNA show that both UMP and UDP inhibit C^{14} uptake. The extent of inhibition depends on the concentration of

nucleotide added. At 0.005 M, UDP inhibits orotic acid uptake by 39 per cent; equivalent concentrations of UMP or uridine reduce C^{14} incorporation by 33 per cent and 24 per cent respectively. It follows that nucleotides can enter the isolated nucleus and that their failure to influence the turnover of C^{14} in nuclear RNA is not due to a lack of their availability at the "turnover" site.

A number of other compounds have also been tested for their ability to promote the loss of C^{14} from RNA previously labeled with orotic acid-6-C^{14}. Figure 6 shows the results of some of these experiments. It can be seen that the free base, uracil, has only a slight effect on the rate of C^{14} "turnover," and that uracil is much less

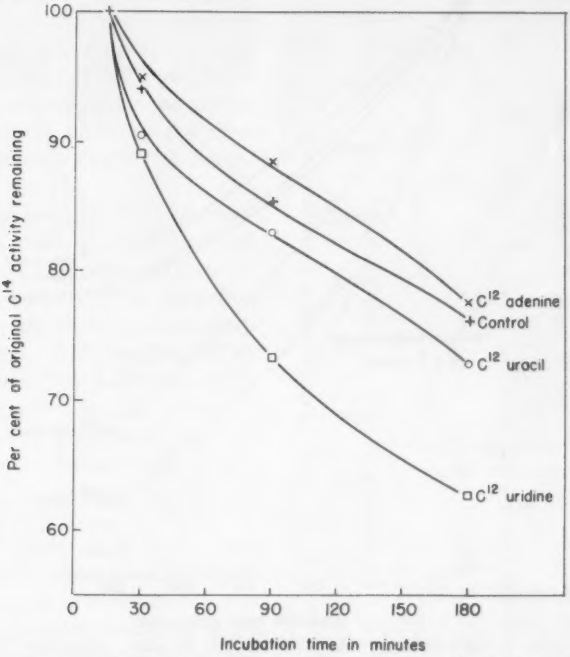

FIG. 6.—Comparative effects of uridine, uracil, and adenine on C^{14} loss from nuclear RNA labeled with orotic acid-6-C^{14}. The incubation medium is given in the legend to Fig. 3. Uridine, uracil, or adenine were added to give a final concentration of 0.005 M.

effective than uridine at the same concentration. An equivalent amount of adenine (0.005 M) does not promote the loss of C^{14} from the nucleus, but may retard it slightly instead.

The effects of three different nucleosides on C^{14} retention in nuclear RNA are compared with that of uridine in Figure 7. It is clear that the purine riboside, adenosine, and the pyrimidine deoxyriboside, thymidine, have no effect on C^{14} "turnover" in this system. The uridine curve shows the expected loss of nuclear radioactivity. Cytidine is about half (56%) as effective as uridine in increasing the rate of isotope loss, but it is not known whether this effect is due to cytidine itself, or whether some deamination of the nucleoside occurs to form uridine.

All of the C^{14} retention curves so far presented were obtained by measuring the radioactivity of the total RNA in acid-washed nuclei.[2] In the tests just described the loss of C^{14} activity from nuclear ribonucleic acid was also verified by measuring the radioactivity of the RNA released from the nucleus by digestion with ribonuclease. Counts of the ribonuclease digests gave results which resemble in all respects the pattern of C^{14} loss summarized in Figure 7.

The Reversible Incorporation of Uridine-C^{14} into Nuclear RNA.—Suspensions of calf thymus nuclei actively incorporate C^{14}-uridine into their ribonucleic acids.

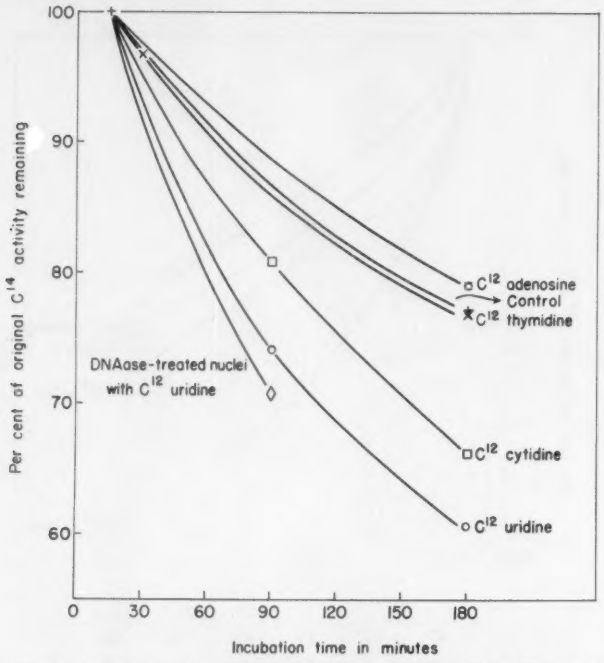

FIG. 7.—Comparative effects of uridine, cytidine, thymidine, and adenosine on C^{14} loss from nuclear RNA labeled with orotic acid-6-C^{14}. The incubation medium is given in the legend to Fig. 3. The nucleoside concentration was 0.005 *M* in all cases. Some of the flasks containing C^{12}-uridine received 1 mg of DNAase. The C^{14} retention in these experiments is shown in the lowest curve.

The time course of uridine incorporation is shown in Figure 8. The ordinate gives the total radioactivity incorporated into the RNA of 40 mg of nuclei after incubation for the indicated times in the presence of 40 μg (24,700 counts) of randomly labeled uridine-C^{14}. (The incubation conditions are given in the legend to the figure.) Evidence that the incorporated C^{14} occurs in nuclear RNA has been obtained in several ways: (1) the RNA has been isolated by the phenol method and found to be radioactive, (2) alkaline hydrolysis releases over 95 per cent of the counts as mononucleotides which can be separated by chromatography on Dowex-1 (see below). Table 1 gives the distribution of C^{14} activity in the cytidylic and

uridylic acid fractions prepared from RNA labeled with C^{14}-uridine. It can be seen that most of the radioactivity appears in uridylic acid; after 30 minutes' incubation the specific activity of cytidylic acid is about 14 per cent of that found in uridylic acid.

The slope of the uridine incorporation curve (24 cpm per minute) corresponds to the uptake of 2.31 μg of uridine-C^{14} per hour. The RNA of the nucleus is 12.5 per cent uridine by weight. Assuming that all the uridine taken up represents RNA synthesis (this is not actually the case) an estimate can be made of the rate of RNA production in the isolated nuclei; the figure obtained is 18.6 μg of RNA per hour per 40 mg of nuclei. This is considerably higher than the rate of synthesis calculated from adenosine-C^{14} incorporation data (13.8 μg of RNA per hour). The higher figure obtained in the uridine-C^{14} experiments is probably the result of increased uptake due to "exchange" reactions (see below).

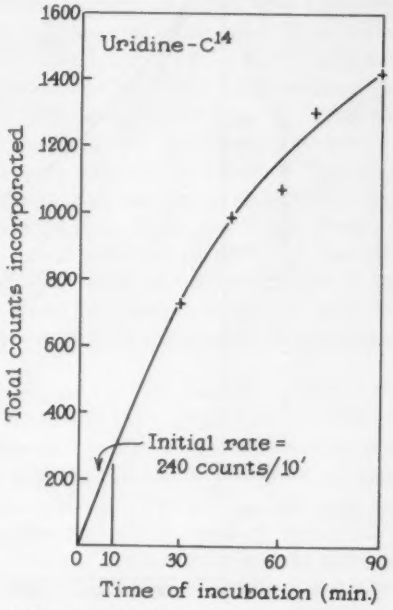

FIG. 8.—The time course of uridine-C^{14} incorporation into the RNA of isolated thymus nuclei. The total counts incorporated into ribonucleic acid are plotted against the time of incubation. The incubation medium is given in Table 1, footnote[b].

Many of the tests for "C^{14}-turnover" carried out on nuclei after orotic acid-6-C^{14} labeling have been repeated with C^{14}-uridine labeled RNA. The procedure used in the uridine experiments is essentially the same as that described before. The nuclei were first incubated in the presence of 40 μg of randomly labeled C^{14} uridine. After 30 minutes at 38°, they were centrifuged down and washed twice with 7 volumes of medium containing a large excess of unlabeled uridine (1.22 mg per ml). The washed nuclei were then resuspended and shaken in C^{14}-free medium for periods of 15 to 180 minutes. The total radioactivity remaining in the nuclear RNA at different times was compared with that present at the outset of the incubation. A plot of uridine-C^{14} retention versus incubation time is shown in Figure 9. The upper curve shows the loss of radioactivity in the "control" nuclear suspensions. The lower curve presents the results obtained on incubation in the presence of unlabeled uridine (0.005 *M*). The middle curve shows the inhibitory effect of sodium cyanide (0.001 *M*) on C^{14}-"turnover" in nuclear RNA. Since cyanide ions are potent inhibitors of aerobic phosphorylation reactions in isolated nuclei, the inhibition here suggests that the C^{14}-"turnover" reaction is also partly dependent on the phosphorylation system.

A special interest attaches to the rate of isotope loss from RNA labeled with C^{14}-uridine. In the previous experiments, using orotic acid-6-C^{14} as a precursor, only the bases of the RNA were labeled. Under these conditions 40 per cent of the counts

were lost in a 3-hour incubation period. When randomly labeled C^{14}-uridine is used as the RNA precursor *both the bases and the sugar* of the pyrimidine nucleotides become radioactive. If the subsequent loss of C^{14} were due only to a "turnover" of a pyrimidine base, then the radioactivity associated with the sugar should remain in the RNA. If, on the other hand, both the sugar and the base are displaced by the "turnover" reaction, the C^{14} activity of the RNA will decrease more rapidly. A comparison of the retention curves for uridine-labeled RNA and orotic-labeled RNA shows that about 40 per cent of the initial activity is lost in both cases (in a 3-hour

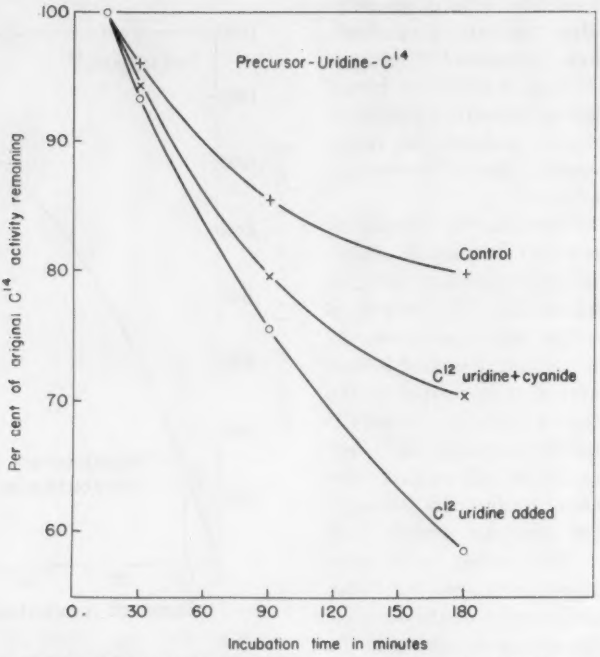

FIG. 9.—The time course of isotope loss from nuclear RNA previously labeled with uridine-C^{14}. The nuclei were labeled as described in Table 1, footnote[b]. The upper curve shows the loss of radioactivity in the medium described in the legend to Fig. 3. The lowest curve shows the C^{14} loss when C^{12}-uridine (0.005 *M*) is added to the medium. In the middle curve the results are plotted for isotope retention in the presence of 0.001 *M* sodium cyanide.

period with uridine present). *Since the rate of C^{14} loss from RNA labeled in both base and sugar is equal to the rate of C^{14} loss from RNA labeled to the base alone, it follows that the C^{14} sugar is not being retained in the nucleic acid molecule, but is lost from it together with the base.*

(It should be mentioned that this conclusion is based on the assumption that the C^{14}-uridine incorporated was not first cleaved to uracil, and the sugar discarded by the nucleus. This possibility (although unlikely in view of the poor utilization of free pyrimidines for RNA synthesis in mammalian cells[18, 19]) has been tested and

found not to occur. Added C^{12}-uracil has no effect on the rate of uridine-C^{14} incorporation into the RNA of isolated nuclei.)

RNA Nucleotide Separation and Analysis.—In order to obtain more specific information about the sites involved in the loss of isotope from orotic acid- or uridine-labeled RNA, the nucleotides were separated by ion-exchange chromatography. The procedure in these experiments followed that previously described, but the scale of operation was increased tenfold in order to provide enough material for convenient separation, UV analysis, and radioactivity measurements of the nucleotide fractions.

Following the labeling of the RNA and the subsequent incubation to permit "turnover," the nuclei were centrifuged down, washed four times with 2 per cent perchloric acid, twice with 3 : 1 ethanol-ether, once with ether, and dried. Two hundred milligrams of the dried nuclei were suspended in 2.5 ml of 0.5 *N* KOH and kept at 38° for 20 hours. This hydrolyzed the nuclear RNA to its component nucleotides. The hydrolyzate was then acidified with perchloric acid to pH 1, and the resulting precipitate (containing the DNA) was discarded. The nucleotide solution was then brought to pH 7 and passed through a column of Dowex-1 (formate). The adsorbed nucleotides were eluted separately from the column by a modification of the method of Hurlbert *et al.*[14, 15] Each nucleotide in the eluate was identified by its ultraviolet absorption spectrum, and then adsorbed on activated charcoal. The charcoal was plated on filter-paper planchets for the determination of the radioactivity of the adsorbed nucleotide. From the amount of material adsorbed and the radioactivity measured, the specific activity of the nucleotides was calculated.

The results are shown in Table 2, which compares the final specific activities of the uridylic and cytidylic acids prepared from RNA with the original activities of these two nucleotides before "turnover" had occurred. In Experiment 1 the retention of the isotope in a "control" nuclear suspension is compared with that observed when the medium contains an excess of unlabeled uridine. (The RNA in this case was labeled with orotic acid-6-C^{14}.) It is clear that the cytidylic acid of nuclear RNA is not losing its C^{14}; on the contrary, it shows an increase in its specific activity. This contrasts with the behavior of the uridylic acid, which loses 40 per cent of its original C^{14} activity in the 3-hour incubation period.

The drop in the specific activity of the uridylic acid fraction during the 3-hour incubation period is not accompanied by any appreciable decrease in the amount of uridylic acid in nuclear RNA. Losses of 3 to 5 per cent have been observed, but the uridylic acid loss in the "control" nuclear suspension has always equalled or exceeded the corresponding loss in nuclei suspended in C^{14}-uridine; yet the RNA in the latter nuclei loses twice as many counts as are lost in the "Controls."

The stability of the cytidylic acid and the instability of uridylic acid in RNA are also evident in nuclei labeled with C^{14}-uridine (see Experiment 2 in Table 2). The third entry in the table lists the results obtained when aliquots of the same nuclear suspension were labeled with either orotic acid-6-C^{14} or uridine-C^{14}. The nuclei were washed free of radioactive precursor, and incubated in the presence of C^{12}-uridine in the usual way. Comparison of the specific activities of the uridylic acid fraction shows that 33 per cent of the original orotic acid was lost in the "turnover" reaction, compared with a loss of 39 per cent of the C^{14} derived from uridine. The

TABLE 2

DIFFERENCES IN ISOTOPE RETENTION IN DIFFERENT NUCLEOTIDES OF NUCLEAR RIBONUCLEIC ACID

Experiment	Isotopic Precursor	Incubation Conditions	Time, Minutes	Adenylic Acid		Guanylic Acid		Cytidylic Acid		Uridylic Acid	
				Specific Activity, cpm/μM	% of Original	Specific Activity, cpm/μM	% of Original	Specific Activity, cpm/μM	% of Original	Specific Activity, cpm/μM	% of Original
1	Orotic-6-C^{14}	Control (1)	15					1,250	100	19,500	100
		Control (1)	180					1,630	131	17,800	91
		C^{12}-uridine added (2)	15					1,210	100	19,800	100
		C^{12}-uridine added (2)	180					1,490	124	12,160	61
2	Uridine-C^{14}	Control (3)	15					224	100	1,220	100
		Control (3)	180					294	131	1,078	88
		C^{12}-uridine added (4)	15					217	100	1,250	100
		C^{12}-uridine added (4)	180					272	125	918	73
3	Orotic-6-C^{14}	C^{12}-uridine added (2)	15					1,210	100	13,430	100
		C^{12}-uridine added (2)	180					1,390	115	9,020	67
	Uridine-C^{14}	C^{12}-uridine added (4)	15					265	100	1,870	100
		C^{12}-uridine added (4)	180					251	95	1,130	61
4	Orotic-6-C^{14}	C^{12}-uridine added (5)	15					1,300	100	8,320	100
		C^{12}-uridine added (5)	180					1,530	117	4,960	60
	P^{32}-Ortho-phosphate	C^{12}-uridine added (6)	15	20,300	100	11,720	100	12,000	100	20,520	100
		C^{12}-uridine added (6)	180	17,480	86	9,960	85	12,270	102	15,270	74

Incubation conditions:
(1) Same medium as in Table 1 (*a*) for uptake phase; no C^{14}-orotic acid present in retention experiments.
(2) As above—medium in retention experiments contains 0.005 *M* C^{12}-uridine.
(3) Same medium as in Table 1 (*b*) for uptake phase; no C^{14}-uridine present in retention experiments.
(4) As above—medium in retention experiments contains 0.005 *M* C^{12} uridine.
(5) Same medium as in Table 1 (*c*) for uptake phase; no C^{14}-orotic acid present in retention experiments but 0.005 *M* C^{12}-uridine present.
(6) As above with P^{32}-orthophosphate in place of C^{14}-orotic acid in uptake phase.

closeness of the results supports the earlier evidence that the "turnover" reaction involves the loss of the sugar as well as the pyrimidine base of the nucleotide. Again, the metabolic stability of cytidylic acid in RNA is evident in nuclei labeled with either orotic acid-C^{14} or C^{14}-uridine.

The nucleotide analyses indicate that the "turnover" reaction in nuclear ribonucleic acid specifically involves its uridylic acid. A further understanding of the nature of the "turnover" can be derived from a consideration of the mode of cleavage of the RNA molecule by alkaline hydrolysis. The internucleotide linkages in RNA are phospho-diester bonds linking the 5′ position of the sugar in one nucleotide with the 3′ position of the adjacent nucleotide. Alkaline hydrolysis cleaves the ester in the 5′ position and releases nearly all of the nucleotides as 3′ (and 2′) phosphates. However, nucleotides joined to the ends of RNA chains by 5′ linkages are released as nucleosides. These pass through the Dowex column before the nucleotide fractions appear. In the analyses reported in Table 2, only the nucleotide fractions were assayed for radioactivity; the nucleosides were discarded. Thus, the changes in specific activity reported for uridylic acid seem to indicate changes taking place *within* the RNA molecule. This would lead to the conclusion that the loss of C^{14} is not merely the result of a "turnover" reaction limited to the 5′ end groups of RNA.

It is, nevertheless, still conceivable that only the terminal uridylic acid in the RNA molecule participates in the "turnover" reaction. If the reaction is considered as a uridylic acid "exchange" the fact that a loss of C^{14}-label is found to occur in non-terminal uridylic acid could be explained by postulating that other nucleotides had been added to the RNA chain after uridylic "turnover" had occurred, thus placing the "exchangeable" uridylic acid *within* the RNA molecule. This explanation presupposes a continuing RNA synthesis which masks the terminal uridylic acid after "turnover" has taken place. This possibility is still under investigation, but some preliminary experiments which tend to support it will be described briefly. Measurements have been made of the rate of C^{14}-loss in nuclei after RNA synthesis has been stopped by the addition of an antimetabolite. Under these conditions, C^{14}-uridylic acid at the 5′ end of the RNA chain should remain terminal, and shielding by the addition of other nucleotides should not occur. If the "turnover" reaction involves the terminal uridylic acids in RNA, more molecules will be available for "turnover" in nuclei in which RNA synthesis is blocked than in the corresponding "controls." Thus, one would expect a greater loss of C^{14} when ribonucleic acid synthesis is inhibited. This was found to be the case. Nuclear ribonucleic acid was labeled with orotic-acid-6-C^{14} in the usual way. The nuclei were washed free of the radioactive precursor, resuspended and incubated for periods of 15 to 180 minutes. To some flasks the antimetabolite DRB (5, 6-dichloro-1-β-D-ribofuranosyl benzimidazole) was added to prevent any subsequent RNA synthesis. (The inhibitor was known to be effective because tests of adenosine-8-C^{14} incorporation in parallel experiments (with and without DRB) showed that RNA synthesis was inhibited by over 85 per cent.) The rate of C^{14}-loss from the RNA of "control" nuclei was compared with that observed in the presence of DRB. After a 3-hour incubation period the "controls" had lost 21.3 per cent of their original C^{14}-activity; the C^{14} loss in the DRB-treated nuclei was 40.1 per cent in the same interval. Higher rates of C^{14} "turnover" were also observed in DRB-treated nuclei in the presence of unlabeled uridine than in the corresponding "controls + uridine." The

results indicate that more C^{14}-uridylic acid is lost when RNA synthesis is blocked. This is the expected result if the "turnover" reaction involves the uridylic acid in terminal positions on the RNA chain, but it does not constitute a proof that the "turnover" is *limited* to end groups in nuclear RNA. Further studies on the site and mechanism of uridylic acid "turnover" are in progress.

So far, only the results of C^{14}-labeling experiments have been presented. They can be explained in terms of a "turnover" reaction specific for the uridine-containing nucleotides of nuclear ribonucleic acid. The tracer evidence that both the base and the sugar are involved in this "turnover" is supported by the finding that the rate of C^{14}-loss is augmented by the nucleoside (uridine) and not by the free base (uracil).

The role of phosphate in this system was also investigated. In these tests the nuclear RNA was labeled by an incubation with P^{32}-orthophosphate. (The conditions were the same as those described in the legend to Figure 9; 2 million counts of P^{32} were added in place of the C^{14}-uridine.) After washing to remove residual P^{32}-orthophosphate, the nuclei were shaken at 38° for three hours. Unlabeled uridine was added to the medium in order to duplicate the conditions for optimum loss of C^{14} from nuclear RNA. Following incubation, the nuclei were washed with 2 per cent $HClO_4$, 3 : 1 ethanol-ether, ether, and dried. The nuclear RNA was hydrolyzed in 0.5 *N* KOH and its component nucleotides were separated and analyzed as described above. Parallel experiments were carried out with C^{14}-orotic acid as an RNA precursor in order to permit a comparison of the rates of C^{14} and P^{32} loss from different nucleotides.

The results are summarized in Table 2, Experiment 4. As expected, the uridylic acid prepared from orotic-labeled RNA lost 40 per cent of its specific activity in the 3-hour incubation period, while the cytidylic acid gained some counts in the same time. The pattern of P^{32} loss in the four major nucleotide fractions is also shown in the table. All of the nucleotides except cytidylic acid show a decrease in P^{32} activity. The significance of this decrease is better understood when it is recalled that alkaline hydrolysis transposes the phosphate from the 5′ position of one nucleotide to the 3′ position of the adjacent nucleotide. In this way, the phosphorus which entered the RNA molecule in the 5′ position of uridylic acid appears in the hydrolyzate attached to any nucleotide which was adjacent to a uridylic acid in the polynucleotide chain. Any loss of P^{32} from the 5′ position of uridylic acid in RNA appears in these experiments as a decrease in the specific activity of nucleotides adjacent to uridylic acid. Losses are evident in adenylic, guanylic and uridylic acids, and it can be concluded that these nucleotides occur in the RNA adjacent to exchangeable uridylic acid. *The "turnover" reaction appears to involve the phosphate radical as well as the uracil and ribose components of uridylic acid.* (It should be pointed out that this loss of phosphate from nuclear RNA in the course of the "turnover" reaction is not readily reconciled with the earlier observation that the rate of C^{14}-loss is increased when unlabeled uridine is added to the medium, but not when UMP is present.)

Nuclear RNA Composition.—The composition of the nuclear RNA in these experiments has been calculated from optical density measurements at the UV absorption maxima of the separate nucleotide fractions. The results of 16 experiments give the average composition:

Adenylic acid	=	10.0
Cytidylic acid	=	14.2
Guanylic acid	=	16.2
Uridylic acid	=	8.5

The sum of adenylic + cytidylic acids (24.2) is very nearly equal to the sum of guanylic and uridylic acids (24.7). Thus, the ratio of 6-amino compounds to 6-keto compounds in nuclear RNA is approximately unity. (See also reference 30.)

Tests were also carried out to see if appreciable amounts of pseudo-uridylic acid[20-22] occur in the uridylic acid peak obtained from the Dowex-1 column in this procedure. The results were negative; there was no shift of the UV absorption maximum to higher wavelengths when the pH was raised from 7 to pH 12, nor was there any decrease in the optical density at 262 mμ. These changes would have been evident if an appreciable amount of pseudo-uridylic acid had been present in the uridylic fraction.

Exchange Reactions in RNA and Nuclear Activity.—What is the significance of the "turnover" of uridylic acid in the RNA of isolated nuclei? A number of attempts have been made to answer this question by relating the "turnover" reaction to other activities of the nucleus.

Many nuclear functions, ranging from the production of ATP to the synthesis of protein and RNA, are impaired by treating isolated nuclei with deoxyribonuclease.[1, 3, 4] A comparison of the rates of C^{14}-loss from the RNA of "control" and "DNAase-treated" nuclei indicates that the rate of the "turnover" reaction is not decreased by treatment with this enzyme (Fig. 7). On the contrary, somewhat more isotope is lost from DNAase-treated nuclei than from the corresponding controls. Since removal of the DNA from the nucleus prevents the further synthesis of RNA,[3] this effect may be similar to the DRB effect already described. In both cases, the inhibition of RNA synthesis means that the C^{14}-uridylic acid at the 5′ ends of the RNA chain would not be masked by the subsequent addition of other nucleotides to the chain. This would make terminal uridylic acid accessible for "turnover" reactions over the entire incubation period.

Reports by Smith and Mills[23] and by Herbert *et al.*[24] have indicated that nuclear fractions isolated in sucrose solutions from liver homogenates are rich in enzymes concerned with the metabolism of uridine nucleotides. One such enzyme, "uridyl transferase," catalyzes the reaction between uridine triphosphate (UTP) and glucose-1-phosphate to form uridine diphosphoglucose (UDPG) and pyrophosphate,

$$\text{UTP} + \text{glucose-1-P} \rightleftharpoons \text{UDPG} + \text{PP}.$$

UDPG plays a role in the synthesis of glycogen[25] and in the conversion of glucose to galactose.[26] On the premise that nuclear ribonucleic acid might serve as a reservoir of uridine nucleotides for use in these and similar reactions, an attempt was made to vary the rate of uridylic loss from nuclear RNA by changing the nature of the sugar in the incubation medium. The results were negative: the rate of C^{14}-uridylic "turnover" in RNA labeled with orotic acid was the same whether the medium contained glucose, or galactose (0.02 *M*), or an equivalent amount of sucrose.

In the study of the optimum conditions for amino acid incorporation in isolated thymocyte nuclei it was found that the sucrose concentration of the medium is an

important variable, and that protein synthesis has a well defined osmotic optimum. Amino acid uptake comes to a halt in hypotonic (0.05 *M*) or hypertonic (0.4 *M*) solutions. Similar effects are observed in the uptake of C^{14}-adenosine or C^{14}-uridine into nuclear RNA. Changes in the sucrose concentration of the medium also affect the rate of C^{14}-loss from nuclear RNA labeled with orotic acid-6-C^{14}. In tests with unlabeled uridine in the medium, the specific activity of the RNA decreased by 34 per cent after a 90-minute incubation in 0.27 *M* sucrose. The corresponding losses in hypotonic sucrose (0.16 *M*) and in hypertonic sucrose solutions (0.49 *M*) were 24 and 12 per cent, respectively. (The incubation media also contained potassium phosphate buffer (0.0125 *M*), glucose (0.02 *M*), NaCl (0.027 *M*), and $MgCl_2$ (0.005 *M*). *The results indicate that the uridylic "turnover" reaction in RNA proceeds best under isotonic conditions. This suggests that this aspect of RNA "turnover" is a normal part of nuclear metabolism.*

Which ribonucleic acids take part in the "turnover" reaction? A partial answer to this question can be obtained by fractionating the nuclear RNA's. We have shown previously that the ribonucleic acid of the nucleus is metabolically heterogeneous, and that the RNA fraction which is extractable in a neutral buffer solution is only one-tenth as active in adenosine or orotic acid uptake as another RNA fraction (insoluble in 1 *M* NaCl) which includes nucleolar ribonucleic acids.[2] This

TABLE 3

C^{14} TURNOVER IN DIFFERENT FRACTIONS OF NUCLEAR RNA LABELED WITH OROTIC ACID-6-C^{14}

Conditions of Experiment	Time	Total RNA		pH 7.1 Soluble RNA		"Nucleolar" RNA	
		Specific Activity, cpm/mg	% of Original	Specific Activity, cpm/mg	% of Original	Specific Activity, cpm/mg	% of Original
Control	15′	7,310	100	923	100	10,330	100
	180′	5,690	77.8	850	92.1	8,140	78.8
C^{12}-uridine added	15′	7,130	100	748	100	10,620	100
	180′	4,000	56	537	71.8	5,490	54

type fractionation has also been applied to orotic acid-labeled nuclei after uridylic exchange had occurred in the RNA. A comparison of the specific activities of the RNA in the two fractions (Table 3) shows that the "nucleolar RNA" has lost 46 per cent of its original C^{14} activity, while the RNA extractable in 0.1 *M* phosphate buffer (pH 7.1) has lost only 28 per cent of its radioactivity. Thus, the "turnover" proceeds more rapidly at the site of extensive RNA synthesis. This aspect of RNA "turnover" may have a special importance in nucleolar metabolism.

This is not the first instance of a loss of C^{14} from ribonucleic acid labeled with orotic acid. While these experiments were in progress Schneider and Potter reported some very interesting observations on C^{14} loss from microsomal RNA in rat liver.[27] In their experiments the microsomal RNA was labeled both *in vivo* and *in vitro* (by incubating liver slices with orotic acid-6-C^{14}). When the isolated microsomes were added to a whole liver homogenate and incubated for 2 to 3 hr., the radioactive nucleotides were lost preferentially. It is especially interesting that this lability was also observed in microsomal RNA labeled *in vivo*. In this case, however, the extent of isotope loss depended on the time elapsed between injection of the C^{14}-orotic acid and the isolation of the microsomes. Microsomes prepared 10 and 30 minutes after isotope injection lost C^{14} from their RNA, but the ribonucleic acid in microsomes isolated 3 or 24 hours after injection did not decrease in specific

activity during the subsequent incubation *in vitro*. This suggests that newly incorporated uridylic acid is especially labile.

The "turnover" of uridylic acid in nuclear RNA may have an analogy in the DNA metabolism of certain cells. Pelc has recently reported a very high rate of thymidine incorporation into the DNA of plant cells. These cells do not subsequently divide, nor do they show a corresponding uptake of labeled adenine or phosphorous into their DNA.[28]

A final point concerns the use of C^{14} uridine in studies of RNA synthesis (e.g.[29]). Considering the specificity of the "turnover" reaction for uridylic acid in RNA, uridine-C^{14} incorporation seems an unreliable indication or measure of RNA synthesis. Our experience with adenosine-8-C^{14} indicates that the uptake of this precursor into nuclear RNA is not followed by any appreciable loss of the C^{14} label. Although this recommends its use in further studies of ribonucleic acid synthesis in the thymus nucleus, it cannot be concluded that incorporated adenosine is equally stable in other systems.

Summary. Nuclei isolated from calf thymus tissue incorporate adenosine-8-C^{14}, orotic acid-6-C^{14}, uridine-C^{14}, and P^{32}-orthophosphate into their ribonucleic acids. Adenosine-8-C^{14}, once incorporated into RNA, is relatively stable and is well retained by nuclei during a prolonged incubation. However, some loss of adenosine-C^{14} from nuclear RNA occurs when unlabeled adenosine is added to the medium.

Orotic acid and uridine are utilized for the synthesis of both cytidylic and uridylic acids in nuclear RNA. Most of the label appears in the uridylic acid. However, much of the C^{14} taken up by nuclear RNA when orotic acid-6-C^{14} or uridine-C^{14} is used as a precursor is rapidly lost on subsequent incubation of the nuclei in C^{14}-free medium. The C^{14} loss is restricted to the uridine-containing nucleotides of nuclear RNA. No corresponding "turnover" is observed in the cytidylic acid.

The C^{14}-turnover reaction involves both the base and the sugar of uridylic acid in RNA. P^{32}-labeling experiments indicate that phosphate is also lost in the process. The rate of C^{14} loss is accelerated by adding uridine to the medium, but the corresponding free base (uracil) or the nucleotide (UMP) do not promote the "turnover" reaction. Some mechanisms are considered.

The "nucleolar RNA" fraction, which is the site of most active RNA synthesis, is also the site of greatest "turnover." Some evidence is presented to show that uridylic acid "turnover" is a normal aspect of nuclear RNA metabolism.

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*LINKED FUNCTIONS IN HEME SYSTEMS: OXIDATION-REDUCTION POTENTIALS AND ABSORPTION SPECTRA OF A HEME PEPTIDE OBTAINED UPON PEPTIC HYDROLYSIS OF CYTOCHROME C**

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Effective interpretation of the relationship between the structure and the biological activity of proteins depends in part on the availability of adequate information about pertinent linked functions.[1] The role of linked functions is especially evident in the chemistry of oxidative enzymes and related conjugated proteins, and among these the heme proteins have long been a subject of intensive study. There exists for the heme proteins a considerable body of experimental data, yet it has proved difficult to arrive at unequivocal conclusions about the detailed meaning of much of the data available. This difficulty reflects in part the deficiencies in our knowledge of protein structure, but reflects also the inadequacy of our knowledge about the linked functions operative in relevant systems of lower molecular weight.

Cytochrome *c* is likely to be among those proteins for which more comprehensive structural information will first become available. Further, the presence in cytochrome *c* of thioether bridges between the prosthetic group and protein components permits the preparation of peptides in which the heme remains tightly bound. The study of such heme peptides may be expected to provide basic information of interest both in relation to cytochrome *c* and in relation to other proteins. The present communication deals with the oxidation-linked proton functions of an undecapeptide prepared by pepsin-catalyzed hydrolysis of beef-heart cytochrome *c*.

Experimental.—Materials: Beef-heart cytochrome *c* was obtained from the Sigma Chemical Company, and was purified before use by ion-exchange chromatography, essentially as described by Paléus and Neilands.[2]

The heme peptide (HP_p)[3] which results from pepsin-catalyzed hydrolysis of beef-heart cytochrome *c* was prepared[4] by the procedure of Tuppy and Paléus,[5] modified in minor details. The product was stored in the dry form, obtained by lyophilization of a solution containing 10^{-3} *M* ammonium hydroxide. Tuppy and Paléus have demonstrated the following amino acid sequence for the peptide portion of HP_p:

Porphyrin

Val-Glu(NH_2)-Lys-Cys-Ala-Glu(NH_2)-Cys-His-Thr-Val-Glu

Quantitative amino acid analysis[6] of the preparation used in the present study yielded the results given in Table 1. The data for lysine, histidine, threonine, glutamic acid, alanine, and valine agree with theory and with the results of Tuppy and Paléus;[5] quantitative results for other amino acids were not reported by these authors. The iron porphyrin was removed from the peptide with the use of performic acid, and the resulting material was then analyzed by the technique of Moore, Spackman, and Stein.[7, 8] Iron analysis[9] yielded a value of 2.95 per cent. Calculated values range from 2.91 per cent to 2.97 per cent, depending on the formula assumed for the lyophilized compound.

TABLE 1

AMINO ACID ANALYSIS OF HEME PEPTIDE

Amino Acid	Residues per Mole of Peptide*	Amino Acid	Residues per Mole of Peptide
Lysine	0.97	Glycine	0.09
Histidine	1.03	Arginine	0
Threonine†	0.85	Methionine sulfone	0
Glutamic acid	2.90	Proline	0
Alanine	1.09	Methionine	0
Valine	2.09	Isoleucine	0
Cysteic acid	0.97	Leucine	0
Half-cystine	0.30	Tyrosine	0
Aspartic acid	0.14	Phenylalanine	0
Serine	0.15		

* The measured values were adjusted to residues per mole of peptide by a constant multiplying factor obtained from the first six amino acids by assuming a composition $Lys_1His_1Thr_1Glu_2Ala_1Val_2$.

† Value not corrected for loss on hydrolysis.

Preliminary observations are reported for samples of HP_p modified by acetylation or by treatment with trypsin. Acetylation was carried out by the addition of 0.1 ml of acetic anhydride to a solution containing 6.5 ml of glacial acetic acid and 6.5 ml of a 1×10^{-4} *M* solution of HP_p. The reaction mixture was maintained at pH 6.6, in an ice bath, and the addition of acetic anhydride was carried out slowly, with stirring, over a period of 30 minutes. At the end of a total of 45 minutes the reaction mixture was subjected to dialysis against water. Preparation of trypsin-treated HP_p was performed essentially as described by Tuppy and Paléus.[5] The product expected[5, 10, 11] is an octapeptide, obtained by the removal from HP_p of the N-terminal sequence Val-Glu(NH_2)-Lys.

Trypsin, pepsin, and catalase were crystalline products of the Worthington Biochemical Corporation. Methyl viologen was obtained from The British Drug Houses, Ltd. All were used without further purification.

The following buffers were employed: acetate, imidazole, phosphate, and borate. Ionic strengths varied from 0.04 to 0.20. All salts were potassium salts.

Methods: The apparatus and procedures used in oxidation-reduction potentiometry were essentially those described in detail in an earlier publication.[12] Spectrophotometry under anaerobic conditions was performed with cuvette assemblies similar to those used in oxidation-reduction work; the insertion of a glass electrode permitted spectrophotometric and pH measurements to be made concurrently under anaerobic conditions. Spectra were recorded with Cary model 11, Spectracord model 3000 and Zeiss model PMQ II spectrophotometers.

Results.—Spectrophotometric Observations: Quantitative study of solutions of HP_p requires a careful control of conditions. Decomposition of the metalloporphyrin group occurs readily, and, as Ehrenberg and Theorell[13] have shown, the system is subject also to pH-dependent aggregation effects. In 5×10^{-4} *M* solutions, HP_p was found to vary in state from monomer at acid pH to predominantly penta- or hexamer at alkaline pH. Polymerization occurs also at consider-

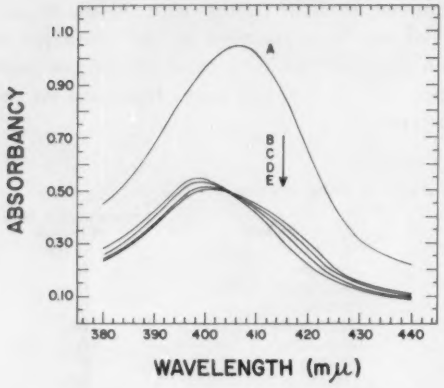


FIG. 1.—Change in the Soret spectrum of a solution of ferri HP_p upon dilution. Phosphate buffer, pH 6.9; room temperature; 1-cm. cuvette for *A*, 10-cm. cuvette for B-E. A, ferri $HP_p \cong 1 \times 10^{-5}$ *M*; *B*, after 20-fold dilution; *C*, same solution 65 minutes after dilution; *D*, same solution 135 minutes after dilution; *E*, same solution 400 minutes after dilution.

ably lower concentrations, and the titration data and spectrophotometric data reported by Paléus, Ehrenberg, and Tuppy[14] both bear evidence of interfering effects. One of the first needs in the present work was the determination of conditions under which a stable, monomeric system could be attained, and the spectrophotometric studies reported here were directed primarily to that end.

Curve A of Figure 1 shows the Soret band of an approximately 10^{-5} *M* solution of ferri HP_p in 0.05 *M* $KH_2PO_4 - K_2HPO_4$ buffer, pH 6.9. Upon 20-fold dilution of this solution, spectrum B was observed. Curves C through E show the subsequent change with time of this spectrum as the solution remained undisturbed in a stoppered cuvette. Greater dilution ratios led to increasingly rapid change in spectrum. In all instances, however, the final position of the absorption maximum was at 398 mμ. An approximately 7×10^{-6} *M* solution brought to a temperature of 50° displayed changes in spectrum qualitatively similar to those shown in Figure 1: the wavelength of maximum absorption diminished with time to 398 mμ. Alteration of the pH of a 7×10^{-6} *M* solution from pH 7 to pH 3 also caused a variation of spectrum with time. In this case, the final absorption maximum was at 395

mμ. These observations have been interpreted as reflecting in each instance the production, by depolymerization, of a single entity with an absorption maximum at 398 mμ at pH 7 and at 395 mμ at pH 3. All other data obtained in the present study, as well as the data of others,[13, 14] are fully consistent with the view that this entity is the monomer of ferri HP_p.

Dilution experiments conducted with ferri HP_p modified by acetylation or by treatment with trypsin gave very different results. The Soret band displayed at pH 7 by 2×10^{-5} *M* solutions of such preparations was found to be at 398 mμ, and this position remained unaltered upon hundredfold dilution to 2×10^{-7} *M*. Full analyses of the modified preparations have not been performed, and these experiments must at present be rated as preliminary only. The results strongly suggest, however, that an amino group plays a key role in the polymerization of ferri HP_p, in agreement with an earlier conclusion of Ehrenberg and Theorell.[13]

The addition of either ammonium hydroxide or imidazole to a solution of ferri HP_p with a maximum at 398 mμ was found to result in alteration in the position of the Soret band from 398 mμ to 406 mμ. This shift is considered to reflect the reaction of nitrogenous base with ferri HP_p monomer to form a compound in which the nitrogenous base occupies coordination position 6 about the heme iron. The experiment illustrated in Figure 1 and the companion studies which have been described all were performed with solutions previously subjected to removal of ammonia present in the lyophilized preparation. This was done either by dialysis or by extensive de-aeration with helium.

The Soret spectra recorded near pH 7 for ammonia-free, 10^{-5} *M* solutions of ferri HP_p proved difficult to reproduce quantitatively. Extensive de-aeration of solutions freshly prepared with lyophilized HP_p led to variable shifts of the Soret maximum to longer wavelengths. These shifts are believed to reflect an increase in the degree of polymerization of the system, following the removal of ammonia from coordination position 6 about the heme iron. Addition of excess ammonium hydroxide to such de-aerated solutions led to the re-establishment of a reproducible maximum at 406 mμ. Addition of imidazole had the same effect. It is assumed that the nitrogenous base reacts with ferri HP_p polymer to form the monomer with the added base in coordination position number 6. Ehrenberg and Theorell[13] previously have shown that the addition of histidine to a concentrated solution of ferri HP_p at pH 8.7 effects a decrease in sedimentation rate. Solutions of ferri IHP_p never displayed alteration in the position of the Soret maximum upon treatment with helium.

In the course of experiments in which solutions of ferri HP_p were subjected to de-aeration with helium or nitrogen, a diminution in absorption spectrum, reflecting metalloporphyrin destruction, was observed. The effect was not attributable to any mechanical action of the gas, for the passage of oxygen through solutions not previously exposed to helium or nitrogen was without effect. Further, decomposition could be observed also when helium or nitrogen simply was passed over the surface of a solution of ferri HP_p, and drawn into the solution with the aid of a vortex created by the action of a magnetically driven stirring bar. Solutions of ferri HP_p stored without the passage of gas showed no spectrophotometric evidence of decomposition.

The degree of destruction could be varied greatly. Ferri HP_p in a 5×10^{-7} *M*

solution at pH 7, kept in an open container, was destroyed virtually completely when helium was passed gently through the solution for 24 hours. Rapid passage of helium through a second sample of solution carefully protected from the atmosphere led to an initial destruction of some 5 per cent, but essentially no further decomposition could be detected upon the continued passage of helium for another 18 hours. Exposure of such a de-aerated solution to oxygen led to renewed diminution in the absorbancy values recorded, the extent of change reflecting the rate at which oxygen was administered. Once full oxygenation was attained, further passage of oxygen was without effect. Decomposition thus clearly was favored by conditions intermediate between those obtaining in fully oxygenated and fully deoxygenated solutions.

The addition of excess imidazole to solutions of ferri HP_p, at values of pH greater than 6, prevented the effects just described. Coordination of a hydroxyl group was

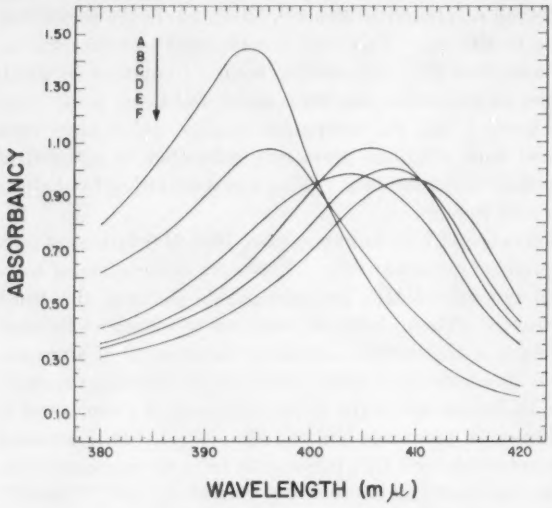


FIG. 2.—Change with pH in the Soret spectrum of a solution of ferri IHP_p. Ferri $HP_p \cong 1 \times 10^{-5}$ *M*, imidazole = 0.05 *M*; no buffer; anaerobic conditions; room temperature; 1-cm. cuvette; titrating agents, KOH and HCl. *A*, pH 1.8; *B*, pH 4.1; *C*, pH 5.1; *D*, pH 7.0 and pH 9.4; *E*, pH 11.6; *F*, pH 12.6.

found to give a similar result: at strongly alkaline pH, solutions of ferri HP_p proved insensitive to changes in degree of oxygenation even in the absence of added imidazole.

In preliminary studies of the variation of the spectrum of ferri HP_p with pH, significant destruction was encountered as the pH was varied through certain ranges. The range from pH 9 to pH 5 appeared particularly sensitive. Solutions used in these studies were at no time exposed to helium or nitrogen, and ranged in concentration from 1×10^{-7} *M* to 1×10^{-6} *M*. The effect has not been examined in detail, and its meaning is at the moment unclear.

Solutions of IHP_p displayed a different behavior. Under aerobic conditions, no loss of metalloporphyrin was observed within the range of pH 5 to 13. When solutions of ferri IHP_p were studied under strictly anaerobic conditions, there was no evidence of instability over the entire range of pH investigated, from pH 1 to pH 13, and all changes in spectrum observed under these conditions were fully reversi-

ble. A few representative spectra of IHP_p solutions, determined under anaerobic conditions, are shown in Figure 2. To preclude any effects of buffer components, unbuffered solutions were employed.

It may be seen from Figure 2 that, except in more alkaline solution, the position of the Soret maximum of ferri IHP_p remains invariant at 406 mμ over the range of pH where the action of imidazole and amino groups as ligands would be most favored. The position of the Soret band of solutions of ferri HP_p (Fig. 3), and of preparations of ferri HP_p modified by acetylation or by treatment with trypsin, also remains invariant in this range. However, the maximum displayed by these solutions is not at 406 mμ but at 398 mμ. It seems reasonable to assume, on the basis of the total evidence presented, that a Soret maximum at 398 mμ reflects the presence of a nitrogenous base in one of the two coordination positions available about the ferriporphyrin prosthetic group of the peptide, whereas a maximum at 406 mμ reflects the presence of nitrogenous base in both of the coordination positions available. A Soret maximum at 396 mμ, observed at appropriate values of pH

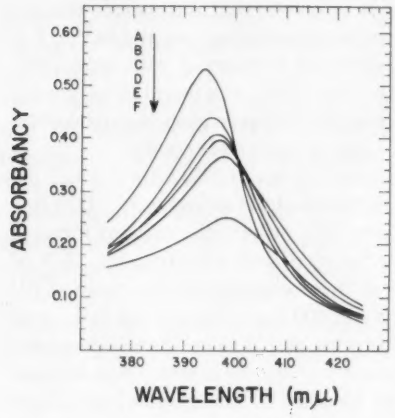


FIG. 3.—Change with pH in the Soret spectrum of a solution of ferri HP_p. Ferri $HP_p \cong 3 \times 10^{-7}$ *M*; no buffer; room temperature; 10-cm. cuvette; titrating agents, KOH and HCl; some destruction, since measurements not made under anaerobic conditions. *A*, pH 2.0; *B*, pH 3.6; *C*, pH 4.4; *D*, pH 5.9; *E*, pH 6.4; *F*, pH 9.9.

with all solutions of oxidized heme peptide studied, might then represent a state in which neither of the available coordination positions is occupied by a nitrogenous base. The same would be true for maxima at wavelengths shorter than 396 mμ, as seen in more acid solution.

The inaccuracy of pH measurements in unbuffered solutions precludes an accurate evaluation of pK′ values on the basis of data such as those in Figure 2, and the degree of overlap between several of the equilibria indicates the need for especial care. Estimated values are: 394 mμ $\rightleftharpoons$ 396 mμ, pK′ $\sim$ 3; 396 mμ $\rightleftharpoons$ 406 mμ, pK′ $\sim$ 4.5; 406 mμ $\rightleftharpoons$ 410 mμ, pK′ $\sim$ 11. A change in molar absorbancy index at 394 mμ occurs at the acid end of the pH range investigated, but measurements have not been extended to sufficiently low values of pH to permit estimation of the midpoint of the change. There is, further, a small change in molar absorbancy index at 406 mμ in the range of pH 6 to 7. It is perhaps of interest to note that the pK′ value estimated for the shift in maximum between 396 mμ and 406 mμ is smaller than that estimated for the shift between 396 mμ and 398 mμ observed with solu-

tions free of added imidazole (e.g., see curves C, D, and E, Fig. 3). This accords with the general experience that the formation of di-nitrogenous base-iron porphyrins is favored over the formation of mono-nitrogenous base-iron porphyrins.

Studies of reduced heme peptide involve a difficulty not encountered with oxidized solutions. The application of reducing agent to any of the oxidized systems thus far examined invariably entailed destruction of metalloporphyrin, presumably as a consequence of peroxide formation. As Tsou first showed,[15] peroxide rapidly destroys HP_p. The effect could be minimized by the careful preliminary de-aeration of solutions with helium, but oxygen adsorbed to glassware cannot effectively be removed in this manner,[12] and peroxide formation occurred to some degree in all experiments. Addition of imidazole or of catalase failed to prevent the effect.

Spectra of ferro IHP_p were determined with solutions first de-aerated with helium and then maintained in a closed system under helium, using the type of apparatus employed in oxidation-reduction potentiometry. Little change in Soret spectrum was observed at values of pH above 7.5, but decrease in pH between pH 7.5 and pH 4 resulted in a marked diminution in absorbancy. Below pH 4, stable solutions of ferro IHP_p could no longer be attained. It is known that ferroporphyrins are subject to loss of iron at values of pH where ferriporphyrins are stable,[16, 17] but whether this is the basis of the instability below pH 4 observed with ferro IHP_p remains to be determined. This instability of ferro IHP_p was found to be greatly diminished in hydrochloric acid solutions of low pH, sufficiently so that oxidation-reduction titrations attempted at pH 1.0 and 1.6 gave acceptable results.

The characteristic "hemochromogen" bands at 551 mμ and 522 mμ are still detectable in spectra of ferro IHP_p determined at values of pH as low as 4. This is in marked contrast to the data obtained with ferro HP_p or with the reduced forms of HP_p preparations modified by acetylation or by treatment with trypsin. A 1×10^{-6} M solution of ferro HP_p displays a typical "hemochromogen" spectrum at pH 8.5, but at pH 6 the spectrum between 500 mμ and 600 mμ indicates that little or no "hemochromogen" remains. Margoliash, Frohwirt, and Wiener[18] have interpreted their data on ferro HP_p as indicating the occurrence of species in which coordination positions 5 and 6 about the heme iron are both occupied by nitrogenous base groups belonging to the peptide moiety of the same molecule. If it may be assumed that, in 1×10^{-6} M solution, ferro HP_p exists predominantly in the form of the monomer, then the data of the present study support this view. Examination of ferro HP_p solutions varying in concentration from 3×10^{-7} M to 2×10^{-6} M revealed no shift in the Soret maximum such as that seen with solutions of ferri HP_p. Possibly the suggested ability of ferro HP_p to form "intramolecular hemochromogen"[18] serves to preclude the polymerization of ferro HP_p over the range of concentrations investigated.

Oxidation-Reduction Potentiometry: IHP_p was selected for a first study of oxidation-reduction potentials, and detailed measurements of the system ferri IHP_p–ferro IHP_p have been completed over the range of pH from 4 to 13. Throughout this range, reduction of ferri IHP_p was fully reversible except for the initial destruction of porphyrin previously described.

Unlike the heme proteins that have been studied, but like other nitrogenous base-metalloporphyrin systems, solutions of IHP_p are "electromotively active," and the use of mediators has not been necessary. Except at very low and very high states

of reduction, equilibrium between electrodes and the ferri IHP_p–ferro IHP_p system was established rapidly. The time required for equilibration varied from seconds to minutes, depending upon the concentration of the solution and the degree of reduction. Concentrations ranged from $1 \times 10^{-5}\ M$ to $4 \times 10^{-5}\ M$.

Sodium dithionite and the semiquinone of methyl viologen[19] were used as reducing agents, and potassium ferricyanide served as the titrating agent in oxidative titrations. The results of both oxidative and reductive titrations agree well with those predicted for a reversible 1-electron oxidation-reduction system in accordance with the relationship:

$$E_h = E_m + \frac{RT}{F} \ln \frac{(\mathrm{Ox})}{(\mathrm{Red})} \tag{1}$$

E_m is the potential of the half reduced system. At pH 0, $E_m = E_o$.

To ascertain whether the variation of potentials observed over the range of pH 4 to 13 reflected fully reversible effects, a number of solutions were each titrated at several appropriate values of pH, and the E_m values so determined were found to agree well with the results of other experiments and with the values subsequently calculated on the basis of equation (2). With two exceptions, measurements were not extended below pH 4 since in this region of pH ferro IHP_p is unstable. The two exceptions are experiments performed in hydrochloric acid solutions of pH 1.6 and 1.0, where ferro IHP_p was sufficiently stable that acceptable data could be obtained. The results of the present series of measurements are summarized in Table 2.

Not listed in Table 2 is the result of a titration of a $1 \times 10^{-4}\ M$ solution of the system ferri HP_p–ferro HP_p, conducted at pH 7.1. The data were, for reasons cited, approximate only. Estimation of the mid-point of the titration curve yields the value $E_m = -0.19$ volt.

The simplest relationship[20] between E_m and pH consistent with the data at hand for IHP_p is that given by equation (2):

$$E_m = E_x + \frac{RT}{F} \ln z \tag{2}$$

where z represents

$$\frac{(\mathrm{H^+})((\mathrm{H^+})^4 + \mathrm{K'_{R1}}(\mathrm{H^+})^3 + \mathrm{K'_{R1}K'_{R2}}(\mathrm{H^+})^2 + \mathrm{K'_{R1}K'_{R2}K'_{R3}}(\mathrm{H^+}) + \mathrm{K'_{R1}K'_{R2}K'_{R3}K'_{R4}})}{(\mathrm{H^+})^3 + \mathrm{K'_{O1}}(\mathrm{H^+})^2 + \mathrm{K'_{O1}K'_{O2}}(\mathrm{H^+}) + \mathrm{K'_{O1}K'_{O2}K'_{O3}}}$$

In the absence of adequate information about the variation of E_m with pH outside the range of pH 4 to 13, equation (2) may be applied only to the range of pH which has been investigated. E_x is the potential which the half-reduced system would have at pH 0, if $\Delta E_m/\Delta \mathrm{pH}$ were constant at -0.120 volt per pH unit between pH 4 and pH 0. $\mathrm{K'_{O1}}$, $\mathrm{K'_{O2}}$, and $\mathrm{K'_{O3}}$ are apparent equilibrium constants for oxidation-linked proton functions in ferri IHP_p. $\mathrm{K'_{R1}}$, $\mathrm{K'_{R2}}$, $\mathrm{K'_{R3}}$, and $\mathrm{K'_{R4}}$ are apparent equilibrium constants for oxidation-linked proton functions in ferro IHP_p. In Figure 4 the experimentally determined values of E_m are plotted against pH and are compared with the curve described by equation (2) upon assignment of the following

constants: $E_x = +0.439$ volt; $K'_{O1} = 2.7 \times 10^{-7}$; $K'_{O2} = 3.4 \times 10^{-11}$; $K'_{O3} = 8.0 \times 10^{-12}$; $K'_{R1} = 2.5 \times 10^{-5}$; $K'_{R2} = 2.5 \times 10^{-6}$; $K'_{R3} = 6.3 \times 10^{-8}$; $K'_{R4} = 1.6 \times 10^{-10}$. Differences between observed and calculated values of E_m are given in Table 2.

TABLE 2

E_m VALUES FOR FERRI IHP_p–FERRO IHP_p SYSTEM AT 30°

pH	Buffer*	Titrating Agent†	E_m Observed, Volt	E_m Calculated, Volt	E_m Observed − E_m Calculated, Volt
1.00	I	A	+0.412	+0.319	+0.093
1.60	I	A	+0.276	+0.247	+0.029
4.04	II	A	−0.040	−0.040	0
4.60	II	A	−0.098	−0.095	−0.003
5.10	II	A	−0.125	−0.131	+0.006
5.67	II	B	−0.159	−0.160	+0.001
6.32	III	B	−0.182	−0.179	−0.003
6.52	III	A	−0.184	−0.184	0
6.93	III	A	−0.197	−0.193	−0.004
7.00	III	B	−0.195	−0.195	0
7.68	III	B	−0.209	−0.207	−0.002
7.72	III	A	−0.208	−0.207	−0.001
8.36	III	B	−0.211	−0.210	−0.001
8.87	IV	B	−0.210	−0.210	0
9.28	IV	A	−0.206	−0.207	+0.001
9.28	IV	C	−0.205	−0.207	+0.002
9.52	IV	B	−0.205	−0.204	−0.001
9.88	IV	A	−0.202	−0.198	−0.004
9.88	IV	C	−0.196	−0.198	+0.002
10.15	IV	B	−0.193	−0.193	0
10.50	IV	B	−0.187	−0.187	0
10.85	IV + V	A	−0.188	−0.187	−0.001
10.85	IV + V	C	−0.188	−0.187	−0.001
11.10	V	B	−0.190	−0.192	+0.002
11.36	V	B	−0.199	−0.199	0
11.95	V	A	−0.222	−0.226	+0.004
11.95	V	C	−0.226	−0.226	0
12.17	VI	B	−0.243	−0.238	−0.005
12.50	V	A	−0.259	−0.257	−0.002
12.50	V	C	−0.253	−0.257	+0.004
12.76	VI	B	−0.282	−0.272	−0.010

* I, HCl; II, $HC_2H_3O_2$-$KC_2H_3O_2$; III, KH_2PO_4-K_2HPO_4; IV, H_3BO_3-KH_2BO_3; V, K_2HPO_4-K_3PO_4; VI, KOH.
† A, semiquinone of methyl viologen; B, sodium dithionite; C, potassium ferricyanide.

Discussion.—The potentials of the ferri IHP_p–ferro IHP_p system are compared in Figure 5 with the corresponding potentials which have been reported for beef-heart cytochrome *c*[21] and for horseradish peroxidase.[12] As may be seen, the potentials of the peptide system differ greatly from those of cytochrome *c*, and resemble more nearly the values recorded for peroxidase. It is of interest that the heme peptide in the absence of added imidazole has been found by Paléus, Ehrenberg, and Tuppy[14] to display, at pH 4.9, 20 times the peroxidatic activity of cytochrome *c*. The results of a single estimation performed near pH 7 indicate that at this pH the potential of the heme peptide in the absence of added imidazole differs little from that found for IHP_p. Minakami, Titani, and Ishikura[22] have reported a potential of −0.3 volt for the imidazole-free system at pH 7. This value is too negative, but the assignment of a potential very much more negative than that of cytochrome *c* is fully in agreement with the results of the present study.

The simplest formulation of the pH-dependence of the ferri IHP_p-ferro IHP_p potentials is that given in equation (2). Oxidation-linked proton functions with

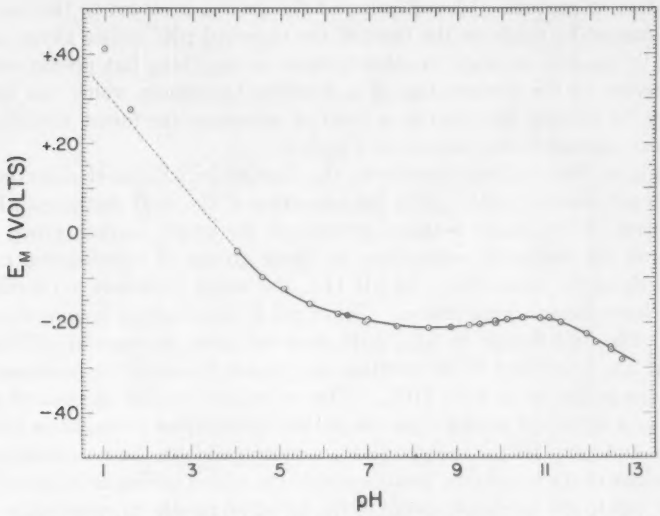


FIG. 4.—The relationship between the oxidation-reduction potentials of the half-reduced ferri IHP_p-ferro IHP_p system and pH. Temperature, 30°. Circled points are experimental values. Four points each represent the results of two experiments, cf. Table 2. The line represents equation (2), with $K'_{01} = 2.7 \times 10^{-7}$, $K'_{02} = 3.4 \times 10^{-11}$, $K'_{03} = 8.0 \times 10^{-12}$, $K'_{R1} = 2.5 \times 10^{-5}$, $K'_{R2} = 2.5 \times 10^{-6}$, $K'_{R3} = 6.3 \times 10^{-8}$, $K'_{R4} = 1.6 \times 10^{-10}$.

pK′ values of 6.6, 10.5, and 11.1 are assigned to ferri IHP_p, and oxidation-linked proton functions with pK′ values of 4.6, 5.6, 7.2, and 9.8 are assigned to ferro IHP_p. These constants of course do not refer to isolated ionization processes. At the least they reflect, besides ionization processes, the changes in free energy attendant upon alteration in the interaction between heme and peptide. Such changes in free energy are apt to differ between one heme peptide or heme protein and another, and, indeed, between reversibly interconvertible states and compounds of a single

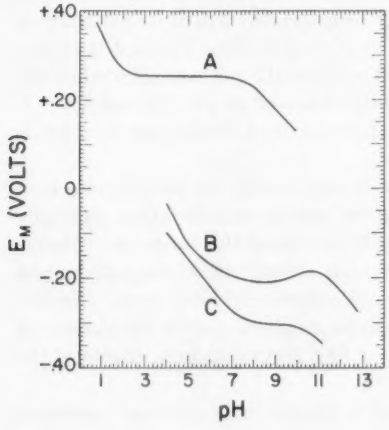


FIG. 5.—E_m-pH curves. *A*, beef heart ferricytochrome *c*-ferrocytochrome *c*[21]; *B*, ferri IHP_p-ferro IHP_p; *C*, ferri horseradish peroxidase-ferro horseradish peroxidase[12].

heme peptide or protein. Identification of the groups involved in the ionization processes cannot be made on the basis of the observed pK′ values alone, nor can reasoning by possible analogy to other systems be anything but precarious. We limit ourselves to the presentation of a working hypothesis which can serve as framework for present data and as a point of departure for future modifications. A schematic representation is given in Figure 6.

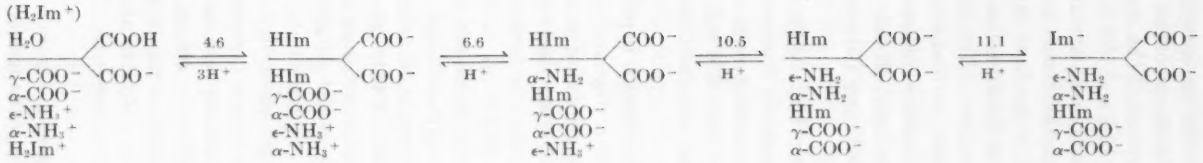
According to this working hypothesis, the changes in $\Delta E_m/\Delta$pH observed upon increase in pH above 6 reflect, with the exception of the final change at pH 11.1, the ionization of the single α-amino group and the single ϵ-amino group of the peptide, and the successive occupation by these groups of coordination position number 5 about the heme iron. At pH 11.1, the added imidazole in coordination position 6 loses its remaining proton. Below pH 6, the situation is somewhat more complex. The first change in $\Delta E_m/\Delta$pH observed upon decrease in pH below 6, that at pH 5.6, is ascribed to the addition of a proton to one of the propionate side chains of the porphyrin in ferro IHP_p. The subsequent change in slope at pH 4.6 is viewed as a net effect arising from the partial cancellation of reactions involving both ferro- and ferri IHP_p. In ferro IHP_p, a proton adds to the remaining propionate side chain of the porphyrin, protons add to the added imidazole in coordination position 6 and to the imidazole group of the histidine residue in coordination position 5, and these two groups are replaced, under anaerobic conditions, by a water molecule in position 6 and by the γ-carboxyl group of the peptide's glutamic acid residue in position 5. In ferri IHP_p, a proton adds to a propionate side chain of the porphyrin, protons add to the added imidazole in coordination position 6 and to the imidazole group of the histidine residue in coordination position 5, and these two groups are replaced, under anaerobic conditions, by a water molecule in position 6 and by the γ-carboxylate group of the peptide's glutamic acid residue in position 5.

The nature of the relationship between E_m and pH at values of pH below 4 is unknown, since the instability of ferro IHP_p in this range of pH precludes oxidation-reduction potentiometry. However, two titrations attempted in hydrochloric acid at pH values less than 2 gave acceptable results, and spectrophotometric observations indicate an enhanced stability of the reduced system under these conditions. No attempt has been made to determine the basis of this increase in stability, but the possibility exists that it may be associated with a coordination of HCl (or a related entity) to the heme iron. It is evident from Figure 4 that continuation from pH 4 to pH 1 of a slope of 0.120 volt per pH unit, as indicated by the broken line, is not in conformity with the data obtained at pH 1.0 and 1.6. It would appear that between pH 4 and pH 2 there occurs a further net increase in $\Delta E_m/\Delta$pH.

It may be noted that no provision is made in this outline for possible effects of buffer components. Such effects must be given serious consideration especially in relation to measurements conducted below pH 5, since in this range the carboxyl and carboxylate groups of the acetic acid-potassium acetate buffers employed might compete for the coordination positions available about the heme iron. Experimental evaluation of this possibility remains to be initiated, and in the absence of evidence to the contrary, we assume that, as a first approximation, effects of the buffer systems employed may be neglected.

The scheme presented appears consonant with the available spectrophotometric

Ferri IHP$_p$



Ferro IHP$_p$

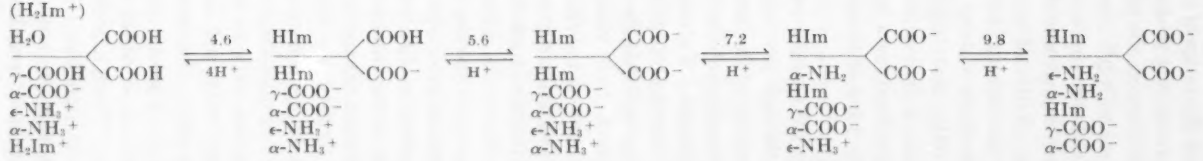


FIG. 6.—A working hypothesis. The iron porphyrin is represented by a horizontal line; the two carboxylate or carboxyl groups attached to this line represent the propionic acid side chains of the porphyrin; the group of the peptide coordinated to the iron porphyrin is shown directly under the horizontal line; the remaining functional groups of the peptide are written beneath this group; the entity occupying the other available coordination position about the iron porphyrin is shown directly over the horizontal line; noncoordinated groups not a part of the peptide are written in parentheses.

data. The changes in ferri IHP_p spectra with pH that are observed in alkaline solution are small compared to those seen in acid solution. The greatest change in alkaline solution occurs in the range of pH where the imidazole group in position 6 is pictured as losing its remaining proton. Here the maximum of the Soret band moves from 406 mμ to 410 mμ as the pH is increased. The degree of overlap between the linked functions with pK'_o values of 10.5 and 11.1 introduces some uncertainty as to a possible change in the Soret spectrum of ferri IHP_p corresponding to pK'_o 10.5, but any such change would involve at most only a slight alteration in molar absorbancy index. Little or no change in the Soret spectrum seems not unreasonable for a reaction pictured as involving the exchange of α- and ϵ-amino groups in coordination position 5 about the heme iron. The linked function with next lower pK′ in ferri IHP_p, that at 6.6, has been ascribed to a process involving an interchange in position 5 of α-amino and histidine-imidazole groups. Again, no great alteration in spectrum need be expected, and all that is observed in the Soret region is a slight decrease in the molar absorbancy index of the 406 mμ band as the pH is decreased.

In marked contrast are the changes seen below pH 6. As the pH of a solution of ferri IHP_p is decreased through the range in which the processes underlying pK'_o 4.6 are operative, the intensity of the Soret band passes through a minimum and the position of maximum absorption shifts from 406 mμ to 396 mμ. Further decrease in pH leads to higher intensities of absorption and to an additional shift to 394 mμ in the wavelength of maximum absorption.

It may be seen from Figure 6 that the reactions reflected in pK'_o 4.6 are assumed to include the displacement, from their respective coordination positions, of the added imidazole in position 6 and of the imidazole group of histidine in position 5. This assumption is founded primarily on the following observations. First, solutions of ferri HP_p, at dilutions at which the monomeric form is the predominant species, display a Soret band with a maximum at 398 mμ at values of pH where an imidazole or amino group of the peptide may be considered to occupy coordination position 5, but a hydroxyl group does not occupy position 6. If the pH of such solutions is made sufficiently acidic, then the Soret band moves from 398 mμ to 396 mμ and the intensity of the band is increased. Further decrease in pH leads, as in ferri IHP_p, to additional enhancement of the molar absorbancy and a shift in maximum from 396 mμ to 394 mμ. More concentrated solutions of heme peptide modified by acetylation or by treatment with trypsin display similar behavior. The 398 mμ band of such solutions moves to longer wavelengths only in regions of pH where a hydroxyl group may be expected to enter coordination position 6. Second, addition at appropriate pH of a nitrogenous base such as imidazole or ammonia to a solution with an absorption maximum at 398 mμ leads to a shift of the maximum to 406 mμ. A gentle stream of helium passed through a solution to which ammonia has been so added results in a shift in absorption maximum from 406 mμ to 398 mμ. Third, a predominant species with a Soret band at 398 mμ is not observed in solutions of ferri IHP_p.

The basis for the remaining assumptions indicated in Figure 6 in relation to pK'_o 4.6 becomes more evident upon consideration of the changes in spectroscopic properties observed with solutions of ferro IHP_p. As with ferri IHP_p, the changes in spectrum observed with solutions of ferro IHP_p at alkaline pH are very much smaller

than those seen at lower pH. The interpretation adopted is analogous to that given for ferri IHP_p and requires no further discussion. Decrease in pH below pH 7.5 leads to an increasingly marked diminution in absorbancy, both in the Soret region of the spectrum and in the region of the characteristic "hemochromogen" bands at 551 mμ and 522 mμ. An obvious possibility is to ascribe this change, associated with pK'_R 5.6, to the addition of a proton to one of the imidazole residues occupying positions 5 and 6 about the heme iron. However, although the absorption in the Soret region of the spectrum continues to undergo great change as the pH is decreased to 4, changes in the bands at 551 mμ and 522 mμ do not keep pace and these bands are still readily seen at pH 4. In the absence of definitive information concerning the possibility that coordinating groups other than nitrogenous bases can lead to the type of spectrum observed at pH 4, the more conservative course is to assume that the added imidazole and the histidine-imidazole group are displaced from their coordination positions not sequentially, but concurrently at a more acid pH as reflected in pK'_R 4.6. The function reflected in pK'_R 5.6 must then be sought elsewhere, and the addition of a proton to one of the propionate side chains of the porphyrin suggests itself as one of the more likely alternatives. The remaining assignments made for pK'_R 4.6 and pK'_o 4.6 have their basis in: (*a*) the premise that the constant for the addition of a proton to a propionate side chain in ferri IHP_p is not apt to differ by more than a pH unit from the corrresponding constant for ferro IHP_p; (*b*) the fact that the potentiometric data indicate a net of one oxidation-linked proton function in ferro IHP_p. The interpretations concerning events below pH 6 clearly represent a most vulnerable part of the present working hypothesis.

Ehrenberg and Theorell have shown[13] that if models are constructed in which the undecapeptide has the form of an α-helix, either the imidazole group of the histidine residue or the ε-amino group of the lysine residue can occupy a coordination position about the heme iron, but only one of these groups can be so coordinated at any one time. Consideration of this fact, in conjunction with the results of studies of concentrated solutions of ferri HP_p,[13] has led to the suggestion by these authors that in solutions of the heme peptide the peptide component does indeed exist in the form of an α-helix. Margoliash, Frohwirt, and Wiener,[18] on the other hand, believe that the peptide does not conform to an α-helix. They have shown that if models are constructed in which the peptide is given an extended configuration, the imidazole group of the histidine residue and the ε-amino group of the lysine residue can at the same time be placed in opposite coordination positions about the heme iron, and they have interpreted their data for solutions of ferro HP_p as indicating that intramolecular "hemochromogen" formation, forbidden by an α-helix, does in fact occur. It may be that the disagreement is, in part, more apparent than real. The experimental data underlying Ehrenberg and Theorell's suggestion pertain to solutions of ferri HP_p. The data upon which Morgoliash *et al.* have based their interpretation pertain to solutions of ferro HP_p. There is no reason to believe that the same situation must obtain in both states of oxidation. The data of the present study have tentatively been interpreted to indicate that in ferri HP_p the imidazole, α-amino and ε-amino groups of the peptide can be coordinated to the heme iron only one at a time: the Soret band of sufficiently dilute solutions of ferri HP_p moves to wavelengths longer than 398 mμ only in

the region of pH where hydroxyl may be expected to coordinate. This interpretation for ferri HP_p is in accord with that part of the suggestion by Ehrenberg and Theorell which indicates that entry of nitrogenous base into both of the coordination positions available about the iron porphyrin requires deviation from the monomeric state. Our data for ferro HP_p, on the other hand, can be construed to be in agreement with the view of Margoliash *et al.*, although here the uncertainties appear greater. Irreversible effects have precluded quantitative dilution experiments with ferro HP_p solutions of sufficiently low concentration, and it has not been possible with our present equipment to measure accurately the spectra between 500 mμ and 600 mμ displayed by solutions of ferro HP_p at concentrations less than 10^{-6} M. The Soret spectra observed with solutions of ferro HP_p ranging from 3×10^{-7} M to 2×10^{-6} M suggest, however, that at these concentrations intramolecular "hemochromogen" formation may well compete favorably with intermolecular "hemochromogen" formation. It is clear in any event that should our present working hypothesis in its major aspects be substantiated by future data, then the conclusion would seem to follow that the configurational state of the peptide component in the systems studied is variable and not fixed.

The studies by Clark and his co-workers on metalloporphyrin-nitrogenous base systems[23, 24] have shown that the chemistry of heme systems of lower molecular weight is not necessarily a "simplified edition" of the corresponding chemistry of related heme proteins. The lower-weight systems and the proteins have their individual complexities. It is too early to say whether the data on the IHP_p system have relevance to the recent suggestion by George and Lyster[25] that in peroxidase and catalase there occur "crevice structures" in which both the fifth and the sixth coordination positions about the heme iron are occupied by groups of the protein, and that the conversion of peroxidase to paraperoxidase may involve a conformational change resulting in the substitution of one coordinating group for another. It is too early also to assess the present data in relation to cytochrome *c*. Theorell and Åkeson[26] have advanced the suggestion that coordination positions 5 and 6 about the heme iron in cytochrome *c* are both occupied by imidazole groups. Margoliash *et al.*[18] believe that the coordinating groups in cytochrome *c* are an imidazole group and an ϵ-amino group. Whatever may ultimately prove actually to be the case, the present data may serve to re-emphasize the fact that the identification of the groups which coordinate to the heme iron in different heme proteins constitutes an essential step, but also only a first step, in efforts to interpret "the extraordinarily delicate adjustment of structure and function in these most versatile of proteins."[1]

Summary.—Studies are reported of the oxidation-linked proton functions of a heme undecapeptide obtained upon peptic hydrolysis of beef-heart cytochrome *c*. Oxidation-reduction potentials of the heme peptide in solutions containing imidazole were measured over the range of pH 4 to 13. The potentials observed differ greatly from the corresponding ones reported for cytochrome *c*, and resemble more nearly those found for horseradish peroxidase. The oxidation-reduction potential of the imidazole-heme peptide system (ferri-ferro) at pH 7 and 30° is -0.195 volt The variation of potential with pH indicates oxidation-linked proton functions with the following pK′ values: oxidized form, 6.6, 10.5, and 11.1; reduced form, 4.6, 5.6, 7.2, and 9.8. Conditions were determined under which various solutions of the

heme peptide appear free of irreversible effects, and the variation with pH of the absorption spectra displayed under such conditions was examined for both the oxidized and the reduced forms of the heme peptide. The relationship between the spectrophotometric observations and the oxidation-reduction data has been considered, and has formed a basis for discussion of the processes reflected in the experimental results reported.

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† Predoctoral Fellow of the National Heart Institute, United States Public Health Service.

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[3] The following abbreviations are used: HP_p, the heme peptide obtained upon pepsin-catalyzed hydrolysis of beef-heart cytochrome *c*; IHP_p, the system HP_p + 0.05 *M* imidazole. The prefixes ferri and ferro are used to designate states of oxidation only and are not intended to indicate a particular electronic configuration.

[4] We should like to thank Dr. Melvin V. Simpson, Dr. Valda M. Craddock, and Mr. Harold M. Bates of this Department for placing at our disposal their experience with the preparation of HP_p and for their helpfulness in the course of the preparative work.

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*AMINO ACID SEQUENCE SELECTION IN PROTEIN SYNTHESIS**

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A living organism is able to synthesize an enormous variety of particular macromolecules—macromolecules of a type which that organism already contains—out of a great variety of different material ("food" in a wider sense). From the point of view of information theory the explanation of this capability of a living organism will be very much simplified if that "food" is considered as being chopped up and then reassembled by a process of replica formation.

We consider the sequence selection in protein synthesis first, before getting into the problem of RNA synthesis, because the assembly of the highly complex and diversified amino acid sequences in proteins calls for an explanation in terms of a general simple scheme; if one can show the possibility of a protein replication scheme which satisfies the physical, chemical, and structural aspects, such a scheme will have evident advantages over other schemes which only too often are overloaded with *ad hoc* postulates.

The selection of a particular sequence of amino acids is one of the main problems in understanding protein synthesis. In the present paper[1] this problem is reduced to the problem of replica formation: The essential information content necessary for the formation of a (filial) protein chain is assumed to be stored in a parental protein chain which is firmly attached to an RNA chain. We shall try to understand this sequence formation as a single step phenomenon rather than as a process involving the subsequent use of hundreds of specific enzymes, synthesizing the chain stepwise, amino acid by amino acid.

In protein synthesis we have to consider all the variety of forces usually involved in biochemical reactions: covalent bonds, ionic bonds, hydrogen bonds; furthermore there is the electrostatic interaction between charged or polar molecules which is strongly modified by gegen-ions from the surrounding ionic medium, and then there are London-van der Waals forces between polarizable molecules.

The latter "polarizability forces" are due to fluctuations of charge distributions in the molecules. Charge fluctuations can be caused by the quantum mechanical zero point motion due to the uncertainty principle. Charge fluctuations may also be due to thermal motion if excited electronic states are in thermal reach. A somewhat different type of thermal charge fluctuations is the fluctuation of proton distributions over the surface of molecules, investigated by Kirkwood and Shumaker. All these charge fluctuation forces have been shown [1, 2] to contribute to an association of like molecules as nearest neighbors which is energetically more favorable than association of unlike ones by an amount of the order of magnitude of a few kT. This property has the important significance that it may account for the selection of the right set of amino acids from the surrounding medium, and for their juxtaposition to the corresponding amino acids of a parental chain.[2] In this paper we shall propose several schemes in which the information about a filial amino acid sequence is stored in an (unfolded) parental protein chain; or, in a separate analysis, one may consider the information stored in a sequence of individual

parental amino acids, independently attached to the RNA chain. In either case the amino acid sequence constitutes the essential part of the "template." The important role of the nucleic acids shall be described later in this paper. In the following schemes we try to leave the task of sequence selection entirely to the specificity property of the charge fluctuation forces without taking recourse to complementarity considerations[3] nor to a multitude of specific enzymes postulated *ad hoc* to accomplish selection of the right sequence.

We do not assume that these filial amino acids (activated amino acids) from the medium will get attracted by their like counterparts in the parental protein at long range from far away; it is Brownian motion which is the agent which shuffles these molecules around rapidly. On the microscopic scale Brownian motion is an extremely rapid phenomenon as the time needed to accomplish a given net displacement is proportional to the square of that displacement. Once filial amino acids come close to the corresponding amino acids in the parental chain, they will be retained there. The selection of the right kind of sequence is achieved in this way.

Some 20 years ago, Hamaker[4] recognized the significance of the equilibrium between static electrostatic repulsions between molecules (in particular between identical molecules) modified by gegen ions (depending on the ionic concentrations in the medium) and the attractive (specific) London-Eisenchitz-Wang (van der Waals) forces. A change of the ionic concentrations in the medium is thus capable of making the nonspecific repulsion predominate over the specific London-van der Waals attraction. Such a change provides for a mechanism for regulating over-all motion of molecules, a motion which might be reversible.

In this paper the question is raised as to how these various forces fit into the process of sequence selection. We want to illustrate the application of the property of specificity of charge fluctuation forces by giving several model schemes. It is evident that the interacting molecules will have to be sterically compatible. In particular, the monomer repeat distances of the nucleic acid chain and of the parental protein chain attached to it should be compatible. It is furthermore necessary to keep in mind that the steric arrangements between filial amino acids which are supposed to be joined by peptide bonds should be such that the peptide bond forming loci would be very close to each other. We definitely want to avoid schemes in which the filial amino acids are many Angstroms apart. The specific interaction due to charge fluctuation forces was discussed in parts I and II. It involves the mutual orientation of the interacting molecules which can be characterized as parallel orientation as regards the z direction (the direction of the line connecting the centers of the two interacting molecules cf. Fig. 3) and antiparallel in the x and y directions. The pictures show this orientation between filial and parental amino acid side chains at several instances. The pictures of space filling models have the purpose of illustrating the spatial arrangements and of showing their compatibility with the steric requirements and the requirements of closeness of interacting loci at the time of peptide bond formation. We have, of course, also to see that the energy conditions for peptide bond formation are properly taken care of. The backbones of parental and of filial chains are parallel oriented. Subsequently, the filial protein chain may be made to peel off from the nucleoprotein template through changes in the ionic constitution of the medium.

As regards the biochemical evidence, the filial amino acids which are to be used

for the synthesis of a new protein, are available in activated form. It is to be emphasized that in our schemes it is quite irrelevant what kind of activation actually is involved. The important steps in the formation of replica proteins are practically the same whether the amino acid activation consists in an ester linkage of an amino acid to the first ribose of a soluble RNA or in a phosphate bond of an amino acid to an AMP, or whether activated small peptides may be the units from which the filial protein chain is assembled. In view of the fact that the detailed steps of the activation mechanism are not yet all settled, we can best perform the molecular model building by using the simplest prototype of activation: we represent an activated amino acid by an amino acid attached, through its carbonyl group, to the phosphate of an AMP, or the monophosphate of another purine or pyrimidine base, i.e., a ribonucleotide.

We further make the usual assumption that the presence of RNA, or DNA, or other substitutes is required before protein synthesis may occur. We discuss RNA schemes; the others might perhaps be similar. We shall use the name RNA when referring to any sequence of nucleotides. In building models, we sometimes use a polyadenylic acid chain or a polyuridylic acid chain, or we use a genuine mixture of nucleotides. The form which the RNA chain or chains will assume and the way of attachment of the parental protein chain (or the parental individual amino acids) to the RNA chain will be discussed along with each of the proposed schemes described in this paper.

This brings us to the concept of a "template." We talk about "templates" even though that word has often been used to designate steric complementarity, complementarity of van der Waals contacts, complementarity of electrostatic patterns of charge distribution, complementarity of hydrogen bond systems—or all of them together. In our case we understand by "template" a molecule or a complex (which contains the proper sequence of parental amino acids, individually or as a protein chain) on which the filial sequence of amino acids is thereupon assembled by specific charge fluctuation forces. The functioning of this template requires the parental amino acid sequence to be arranged so that it forms some kind of a linear array of exposed parental amino acid side chains to which the filial amino acids have access. This arrangement may be achieved by an attachment of the parental sequence to a nucleic acid chain or helix which has the function of a backbone to hold the amino acid sequence, or protein chain. The specific function of the nucleic acid will be discussed below. It may be well to remember that highly charged groups like the phosphates of the nucleotides are probably well neutralized by an additional chain of basic amino acids (as found in microsomal nucleoproteins), by Mg^{++}, or other small ions. The charges on the nucleotides and on the parental and filial amino acids are therefore not expected to completely dominate the mode of attachment in question.

The problem of protein synthesis is really a twofold one: the problem of formation of a template and the problem of formation of a replica protein. In discussing the possible models we concentrate on the latter of these two processes because the schemes of formation of a template are likely to be more dependent on detailed experimental results (for which we still have to wait) than are the schemes of formation of filial protein chains.

In discussing the formation of a replica protein we give, in this paper, detailed

attention only to schemes in which the template is an extended parental protein chain attached to a single strand extended RNA chain by hydrogen bonds (denoted by H). There might be similar schemes involving double strand RNA. There may also be schemes in which the template is a sequence of individual amino acids, attached to the extended (single or double strand) RNA by covalent bonds (denoted by C).[5] Nature provides for an exceedingly accurate conservation of information. This seems to imply that the information content either be preserved by virtue of the parental protein chain remaining tightly hydrogen bonded together with the RNA (in schemes H), or be preserved by virtue of the individual parental amino acids being covalently bound to the RNA chain (in schemes C). In any case we would like to regard nucleoproteins rather than nucleic acids as carriers of genetic information.

Considering the various possibilities of template formation from an RNA chain and a parental protein chain or a parental sequence of individual amino acids, and considering only those sections of the RNA chain which carry parental material, the question evidently arises: what fraction of the set of parental amino acids is attached to what fraction of the set of nucleotides? We will discuss in detail only schemes in which every monomer of the protein chain is attached to the RNA chain. On the one hand that includes the case where the ratio of the number of amino acids to the number of nucleotides in the template is one to one (all the schemes listed in reference (5)). This 1:1 ratio for the template, formed by H bonds between RNA and peptide chain, is supported by the experiments of E. T. Bolton and his co-workers[6] and other groups. On the other hand, every amino acid might perhaps be attached to every nucleotide pair in the wide groove of a Watson-Crick-Rich RNA double helix ($H^{+}{}_{B}2p$), a double helix assumed to be similar to the Watson-Crick-Wilkins DNA helix.

These attachments between protein and RNA chains imply fairly stringent condition as regards the repeat distance of attachment sites on the RNA: they have to match the repeat distance along the protein chain (schemes H). The corresponding conditions in schemes C would not be less stringent: in either case the arrangement of the parental amino acids has to be such that the filial amino acids have a chance to become assembled at the proper peptide repeat distance from each other, i.e., some 3.4 Angstrom apart. Otherwise peptide bond formation in the filial chain could only occur simultaneously with the filial chain peeling off and that seems to be most unlikely to result in a complete filial protein chain.

These schemes may help the biochemist to select the actual process which is at work at protein synthesis. They also pose a number of experimental problems, the solution of which will decide in favor of one or against another of the hypotheses made in regard to protein synthesis theories.

Protein Chain Hydrogen Bonded to Riboses of Single Strand, Stretched-out RNA ($H^{+}{}_{R}1a$).[5]—In this scheme we assume the presence of a single chain RNA molecule whose bases are stretched out alternately from the midline of the chain like the oars of a racing boat. With models it is possible to build an RNA chain with a monomer repeat distance equal to that of an extended protein chain and having the $C_2'OH$ groups on the riboses accessible all from one side as shown in upper Figure 1 (top view). We also assume a protein chain in unfolded form with its carbonyl groups pointing alternately to somewhat opposite sides of the chain's

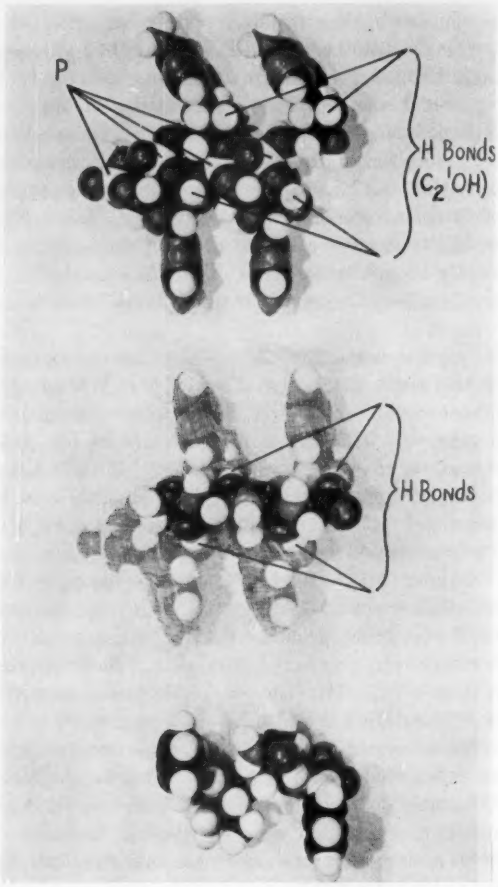

FIG. 1.—All three pictures are "top views" showing the ingredients for the template for H^+_R1a. The upper picture shows the bare four monomer RNA chain with the OH groups on the C'_2 of the four riboses clearly exposed. The middle picture shows this RNA under a veil; over the veil, a four monomer protein backbone is hydrogen bonded to the four C'_2OH of the RNA. The veil makes it possible to distinguish the protein backbone from the RNA. The lower picture shows the protein chain in the same view as the protein backbone of the middle picture; the backbone is mostly hidden behind the side chains. From left to right these are phenylalanine$^+$, valine, glutamine, tryptophane$-$. The same sequence is used in Figures 2, 3, 4, 8, 9, 10.

C is black; H is white, an almost complete sphere, except in an H bond where it is about a cylindrical disk; P is beige, appearing white, tetrahedral: O is red, appearing as a darker gray; double bond O is shaped like a flattened sphere with two indentations, single bond O looks more like a sector of a sphere; N is blue, appearing as a lighter gray.

midline as shown in middle Figure 1 (repeat distance along the midline axis is 3.4 Å). A short but fairly bulky section of the glucagon chain, i.e., tryptophane$^-$, glutamine, valine, and phenylalanine$^+$, is used to illustrate this protein chain, lower Figure 1. The attachment of the protein chain to the nucleic acid chain is through hydrogen bonds between the oxygens of the carbonyl groups of the peptide backbone

and the hydrogens of the OH groups on the C_2' of the riboses of the RNA. Such an attachment would not be possible with DNA. The negative charges on the phosphates may be compensated by Mg^{++} ions and by some basic protein side chains which can get at the phosphates. The RNA with attached (parental) protein chain would act as a template for the synthesis of a filial protein chain. Figure 2 shows this template, its RNA part again being covered by a veil (side view).

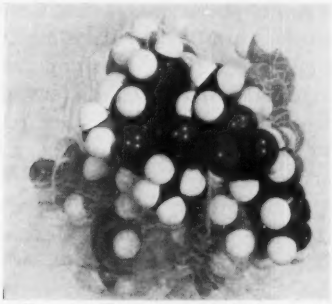

FIG. 4.—Top view of filial protein chain lying on top of the template which is altogether covered by a veil, H^+_R1a. Backbone of filial chain (four oxygens) is visible on top. Relative position of filial to parental protein chain is like that of Figure 10 which is an end view of the chains, showing the filial and parental tryptophanes after the filial chain peeled off.

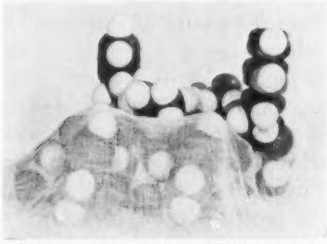

FIG. 2.—Template for H^+_R1a seen in side view. RNA is under the veil, the protein chain of Figure 1 is over it, again tryptophane$^-$ on the right.

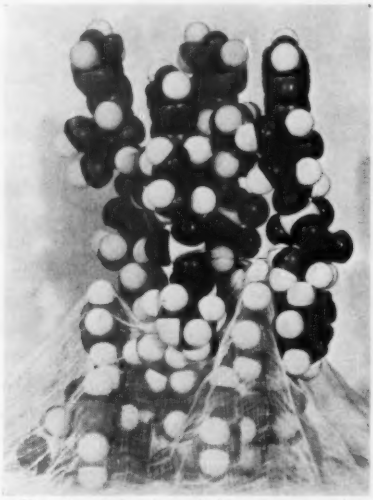

FIG. 3.—Side view of the sequence selection of filial amino acids in H^+_R1a. The template of Figure 2 is entirely covered by a veil; four activated amino acids (AA-AMP), i.e., filial amino acids, lie, in proper orientation, side by side next to the respectively identical parental amino acids on the template, held on by specific charge fluctuation forces, after Brownian motion has brought a variety of amino acids close by. The rear row (phenylalanine and glutamine) of the activated amino acids are represented shaded to distinguish them from the others.

We assume activated amino acids to be available in the medium, in the case of the illustration simply amino acids phosphate bonded to AMP. Four of those activated amino acids are seen in Figure 3. They are retained next to the corresponding parental amino acid side chains, in proper orientation, after Brownian motion has thoroughly shuffled them around. In this Figure 3 (again, a side view), the entire template of Figure 2 is covered by one veil; the activated amino acids are bare.

Brownian motion also brings an amino group of one activated amino acid frequently near to the carbonyl group of the adjacent activated amino acid. The polarity of the filial chain is parallel to that of the parental chain. At this instant

the bonds connecting the amino acids to the ribonucleotides may be broken with simultaneous formation of peptide bonds between the filial amino acids. When the broken-off ribonucleotides have drifted away, the filial protein chain lies attached, as shown in Figure 4, to the parental protein chain. A later change in ionic constitution of the medium permits the filial chain to peel off, as in Figure 10.

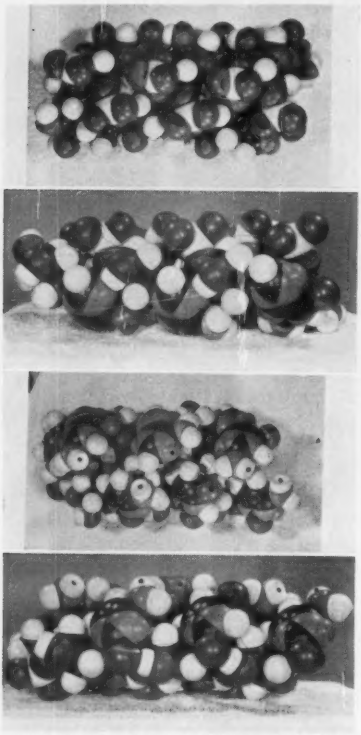

FIG. 5.—Single strand RNA. The four views are related to each other as four views in an engineering drawing. The internal hydrogen bonding is where the disk shaped H bond hydrogens connect between riboses and bases. Figure 6 whose top corresponds to the right-hand side of Figure 5 shows the details. The monomer repeat distance is about 3.3 Å. The sites where the protein chain of Figure 7 is hydrogen bonded to this RNA chain are marked by black dots.

One has also to envisage the possibility of other processes following Figure 3. When the high energy phosphate bonds of the AA-ribonucleotides are broken, there might be the possibility that, besides the formation of a protein chain, an RNA chain is formed, or that a ribonucleoprotein results.

Protein Chain Hydrogen Bonded to Phosphates of Single Strand, Stretched-out RNA ($H^-{}_P1a$).—One can arrange the RNA in a slightly different fashion, with its phosphates in quite an exposed position, all on one (upper) side of the chain, so that the RNA has a phosphate backbone resembling the row of heads of the oarsmen of a racing boat and the bases like the oars which in this case are halfway pulled in. Such an RNA (or DNA) single chain may attain a monomer repeat distance as short as of the order of magnitude 3.2 Å and may have all its phosphates accessible for hydrogen bond attachment to the NH groups of a protein chain. (If the NH groups of a protein chain are turned all more or less toward one side, the side chains pointing to the other side, one has a repeat distance of 3.2Å to expect for the peptide monomers.) In this case $H^-{}_P1a$ it is the oxygens on the phosphates which accept the protein hydrogens: Mg^{++} and some basic proteins might again neutralize negative charges on the phosphates. DNA might function according to the same scheme.

Protein Chain Hydrogen Bonded to Bases of Single Strand RNA with Alternate Base Arrangement ($H^{\pm}{}_B1a$).—The single strand RNA discussed in the first instance ($H^+{}_R1a$) had its bases outstretched, alternately from one and the other side of the phosphate backbone. The next scheme ($H^-{}_P1a$) brought the bases closer to the backbone. The present scheme ($H^{\pm}{}_B1a$) brings the bases very close together. Their closest packing is seen in the illustrations (Fig. 5). The phosphates again form a zigzag backbone with indentations between them which make easy access to the side chains of some (nonspecific) basic protein

and to Mg^{++} ions which compensate the negative charges on the phosphates. This RNA single chain is very compact and has a monomer repeat distance (measured along the midline) of about 3.3 Å. The $C_2'OH$ (which evidently exists only in RNA, not in DNA!) of every ribose forms a hydrogen bond to the structurally adjacent base[7] which is the second-to-next base along the sequence of nucleotides (Fig. 6). The H bond acceptors are the N_7 in case of purines and the C_2O in case of pyrimidines. On the side of the bases there are hydrogen bonding sites, some H donors, some H acceptors to which a protein backbone may be attached (black dot

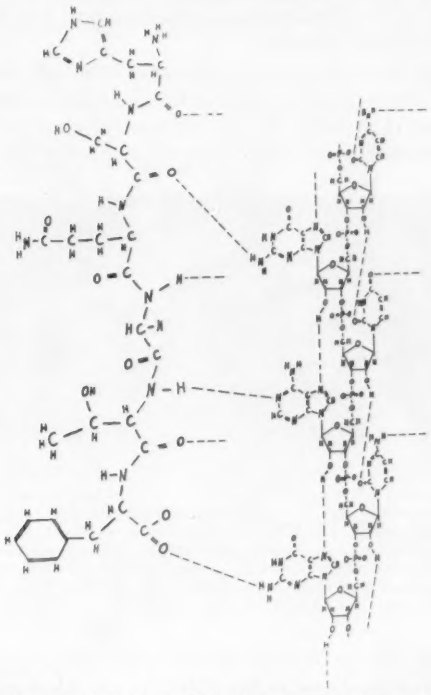

FIG. 6.—Template $H^{++}{-}{-}{+}{+}_{B}1a$. The six hydrogen bonds between protein and nucleic acid are marked by dashes, three of which are interrupted in order not to disturb the RNA formula. The protein chain consists of the first six amino acids of glucagon, i.e., histidine$^+$, serine, glutamine, glycine, threonine, phenylalanine.$^-$

markings in Fig. 5 and 7). But such attachment is conditional on an adequate sequence of nucleotides, in particular when the protein's side chains are taken into account. These side chains not only influence the possible shapes of the protein backbone: their bulkiness also implies direct compatibility conditions with the available space on the rough terrain which the bases of the RNA have to offer. The degree of specificity between this RNA and the parental protein compatible with it will be discussed in a later note.

This compatibility condition between RNA chain and parental protein chain is not of such a kind that three adjacent nucleotides would determine one amino acid

(Gamow's overlapping code) but something related to that coding idea: the compatibility condition is much more relaxed than Gamow's. But it implies that if a foreign nucleic acid is injected into a cell, it will in general no longer be possible to attach the cell protein to that foreign nucleic acid, and a rearrangement of peptide chains into a new parental protein chain might come about which then produces that same new type filial protein. The phenomenon of transforming principle might in this way be understood to operate. According to that view the new protein produced will not only be dependent on the kind of nucleic acid injected but also on the type of protein present in the original cell. (This of course presupposes that the injected transforming principle does not carry any information-containing nucleoprotein with it.)

In this connection, where we have a stereospecific attachment between nucleic acid and protein, we are to be reminded that the degree of conservation of information lies in the degree of stability of that tightly hydrogen bonded nucleoprotein structure, i.e., the template (and in its accurate replication which we have so far

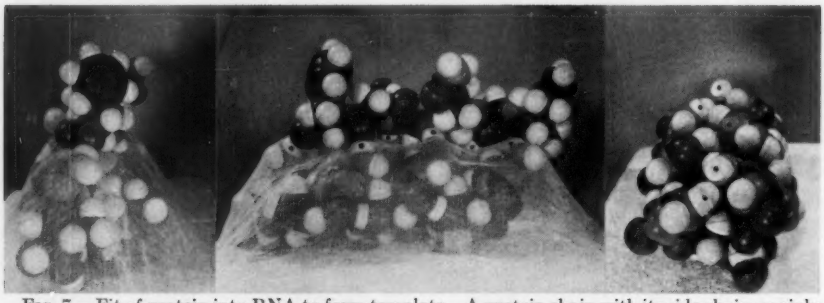

FIG. 7.—Fit of protein into RNA to form template. A protein chain with its side chains mainly pointing in the upward direction, has an average amino acid repeat distance of 3.2 to 3.5 A; it can be shortened, but not lengthened. The RNA structure is very compact, and can, given the internal H bonding, and in view of its compactness, not be shortened as long as it is assumed to be a straight rod. The fit, in the present example, is perfect; in the general case both protein and nucleic acid have about 3.3 A as their average monomer repeat distance. The fit is conditional on the detailed sequence in the two kinds of molecules.

sidetracked). We are also to be reminded that mistakes in synthesis of filial protein chains are of a random character and therefore much less critical than a change in a nucleoprotein which is a highly permanent structure.

Protein Chain Hydrogen Bonded to Bases of Single Strand, Stacked-up RNA ($H^{+}{}_{B}1p$ *and* $H^{-}{}_{B}1p$).—To complete the survey, two schemes should be mentioned. In these schemes the RNA chain is single strand, and assumed to be of the general form of a half of a Watson-Crick-Rich double helix. The function of the RNA is again to provide for a structure on which the stretched-out parental protein chain may be held by hydrogen bonds. A variety of attachments might be possible between either the CO or the NH groups of the protein chain to several sites on the bases of the RNA. The molecule model $H^{+}{}_{B}1p$ shows H bond attachments of the CO groups of the peptides to the NH_2 groups of the adenine bases: the ingredients on Figure 8, the template on Figure 9, and the template with the filial protein chain nearby, after it has been peeled off (Figure 10). The scheme $H^{-}{}_{B}1p$ is analogous: the protein's NH attach to the uracil's bases to their O atoms.

The negative charges on the phosphates are again assumed to be compensated. Other attachments of a protein chain to this nucleic acid chain, monomer by monomer, fail because of the high repeat distance between riboses or between phosphates. DNA would permit a similar scheme.

Double Attachments of Protein Chains.—It does not seem possible that a peptide chain would be hydrogen bonded to an RNA chain by hydrogen bonds both over the NH and the CO groups of the same monomers. Such an otherwise interesting bonding would imply serious obstruction to amino acid side chains.

Covalent Attachments of Individual Parental Amino Acids to the RNA Chain.—Space does not permit elaborating on those schemes. The more important schemes

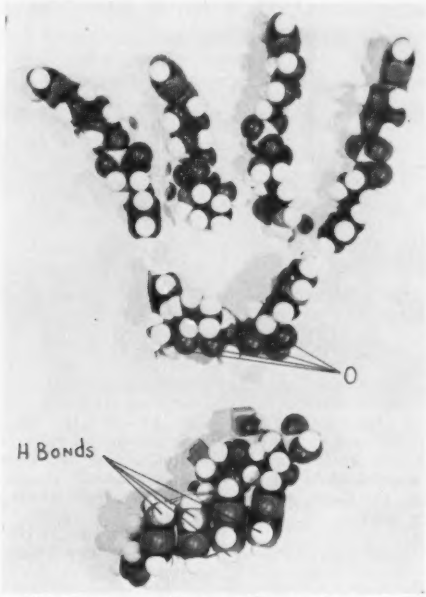

FIG. 8.—Ingredients for process $H^{+}{}_{B}1p$. Upper picture shows four activated filial amino acids; middle picture, the parental protein chain in side view; lower picture, the RNA chain (half of Watson-Crick-Rich double helix) in top view; the axis of this helix is about perpendicular to the four base planes. Bases with H bonds are all visible.

seem to be C_R1a, C_P1a, C_B1a, C_B1p, C_B2p, C_R2p, C_P2p. The latter two would imply attachment, in a zigzag fashion, of one sequence of amino acids in the narrow groove of a Watson-Crick-Rich helix, to all riboses or to all phosphates respectively; the filial protein chain would come to lie along the narrow groove. C_B2p might perhaps be a possibility though not a sterically very clean one, where the amino acids are attached to the bases in the wide groove of a Watson-Crick-Rich helix with the corrected helix parameters given by Wilkins. The first four of these schemes are analogous to the corresponding hydrogen bonded schemes which we have described.

Further Remarks.—It is the purpose of this paper to bring the current biochemical

findings in the field of protein synthesis into proper relation to the physical phenomenon of specificity of charge fluctuation forces so as to account for the right sequence selection of amino acids.

Protein synthesis involves (1) not only formation of a protein chain of proper amino acid sequence, it also involves (2) formation of α helices or sheets, and (3) folding of the helices or sheets into a three dimensional globular or other structure. Only the first of these steps has been discussed in this paper. The second step seems not to raise too difficult problems: an α helix might be easily formed from a chain. As regards the third step, the folding of an α helix into a three dimensional structure may be determined by the sequence of amino acids of the α helix. Or, this folding may be an assembly process of the type described in Figure 5 of our symposium article.[2] Or, it may be determined by other biologically important macromolecules in the surrounding medium.

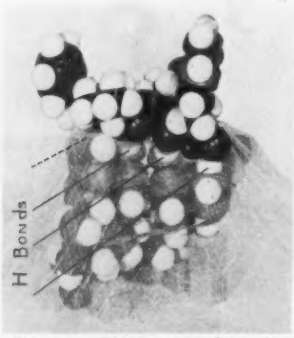

FIG. 9.—$H^{+}{}_{B}1p$ template in side view, RNA under, protein over veil. Three of the four H bonds are visible. Axis about perpendicular to bases, phosphates mostly hidden.

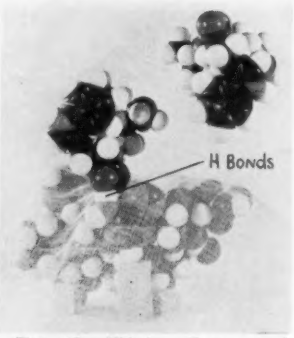

FIG. 10.—$H^{+}{}_{B}1p$. Same as in Fig. 9, but in end view, i.e., in the direction of the RNA axis. The ribose phosphate chain forms a right-handed helix which shows up as a segment of a circle. The filial protein chain, already peeled off, is seen in proper orientation.

The question might arise whether α helices could replicate without unfolding, or not. Suppose a parental α helix has collected filial amino acids from the surrounding medium, in a mantle region surrounding the helix, corresponding to the stage represented by Figure 3. Consecutive amino acids are now, however, very far apart along a helix surrounding the parental helix, and peptide bond formation might come about between amino acids number 1, 5, 9, 13, . . . rather than between number 1, 2, 3, 4, 5, . . . as it should. This clearly does not permit an orderly replication of a protein.

This investigation received important help from many colleagues to whom I would like to express my appreciation and thanks. The work could not have been done without the molecule models for which I am greatly indebted to Dr. Linus Pauling, Dr. Robert B. Corey, and Dr. Richard E. Marsh. At Nebraska I owe thanks to Dr. Robert B. Johnston, Dr. J. R. Matton, Dr. Patricia Weymouth, Miss Audrey Fosbrooke, and Mr. Donald E. McArthur who contributed many ideas in connection with the model building.

* Research supported by the Research Corporation and the National Cancer Institute (National Institutes of Health grant C-3304 BBC), and by the University of Nebraska Research Council.

† Permanent address.

[1] We shall refer to this note as part III. Part I by Yos, Jerrold M., William L. Bade, and Herbert Jehle, these PROCEEDINGS, **43,** 341 (1957); part II by the present author, these PROCEEDINGS **43,** 847 (1957), "Specificity of Intermolecular Forces due to Charge Fluctuations."

[2] Muller, H. J., *Am. Naturalist,* **56,** 32 (1922), *Cold Spring Harbor Symp. Quant. Biol.,* **9,** 290 (1941), and *Proc. Roy. Soc.,* **B 134,** 1 (1947); Haurowitz, F., *Chemistry and Biology of Proteins,* (Acad. Press, 1950), p. 340 ff.; Hamaker, H. C., *Physica,* **4,** 1058 (1937); Jehle, H., J. M. Yos and W. L. Bade, *Phys. Rev.,* **119,** 793 (1958); Yos, J. M., *Phys. Rev.,* **119,** 800 (1958); Yos, J. M., W. L. Bade, and H. Jehle, *in Symposium on Molecular Structure and Biological Specificity,* ed. L. Pauling and H. Itano, (Am. Inst. of Biol. Sciences, 1957), and *Proceedings of the First National Biophysics Conference* (Yale University Press, 1959).

[3] Cf. the remarks in part IV.

[4] Hamaker, H. C., *Rec. Trav. Chim. Pays Bas,* **56,** 3 (1937); *Chem. Weekblad,* **35,** 47 (1938); Overbeek, J. Th. G., in Kruyt, H. R., *Colloid Science,* (Elsevier, 1952) **1,** pp. 276, 277; Derjgauin, B., and L. D. Landau, *Acta Physicochim. USSR,* **14,** 663 (1941); Lifshitz, E. M., *JEPT,* **2,** 73 (1956); Debye, P., and E. Hueckel, *Phys. Z.,* **24,** 185 (1923); Onsager, L., *Chem. Revs.,* **13,** (1933); Klotz, I. M., in *The Proteins,* ed. Neurath and Bailey (Academic Press, 1953), p. 727 ff.; Prigogine, I., and A. Bellemans, *Disc. Faraday Soc., Nonelectrolytes,* **15,** 80 (1953).

[5] $H^{+}{}_{R}1a$ stands for "protein chain *H*ydrogen bonded to the $C_2'OH$ of the hydrogen donor (+) *R*iboses of a *single* (*1*) strand RNA whose bases are stretched out alternately left and right from the phosphate-ribose backbone, like the oars of a racing boat." C_P2p stands for "individual amino acids *C*ovalently bonded to the *P*hosphates of a *double* (*2*) strand RNA, the bases of each strand being stacked-up parallel as in a Watson-Crick-Wilkins-Rich helix." The schemes to be considered are: $H^{+}{}_{R}1a$ protein H bonded to H donor riboses of single strand alternate base RNA, $H^{-}{}_{P}1a$ protein H bonded to H acceptor phosphates of single strand alternate base RNA, $H^{\pm}{}_{B}1a$ protein H bonded to H donor or acceptor bases of single strand alternate base RNA, $H^{+}{}_{B}1p$ protein H bonded to H donor bases of single strand parallel base RNA, $H^{-}{}_{B}1p$ protein H bonded to H acceptor bases of single strand parallel base RNA. The first, third, and fourth of these schemes are represented in the illustrations.

[6] I am indebted to Dr. E. T. Bolton and his colleagues in the Department of Terrestrial Magnetism of the Carnegie Institution, Washington, D. C., for telling me about the not yet published results which they obtained. They have found a ratio of two amino acids per nucleotide in ribonucleoproteins from *E. coli,* and by means of a strong Na^{+} solution they have been splitting off one protein chain, the remainder being constituted in a protein-RNA chain with one amino acid per nucleotide. Subsequent treatment with urea resulted in splitting off the second protein chain from the RNA.

[7] I am indebted to Dr. Chr. Jardetzky who has drawn my attention to this stabilizing hydrogen bond.

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*PYRIDOXAL KINASE OF HUMAN BRAIN AND ITS INHIBITION BY HYDRAZINE DERIVATIVES**

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Although the mechanism of conversion of vitamin B_6 to pyridoxal phosphate is not fully known for any tissue,[1] it requires in every case participation of a kinase. Such kinases must be of almost ubiquitous occurrence and, indeed, have been detected in brain,[2] liver,[3] *Streptococcus faecalis,*[4, 5] *Escherichia coli,*[6] and yeast[6]. Despite the

central role of the ultimate product, pyridoxal phosphate, in amino acid metabolism only the kinase from yeast has been purified significantly and its properties studied in reasonable detail.[6] During a comparative study of pyridoxal kinases from different organisms,[7] brain tissue was found to be among the richest sources of this enzyme. This finding was of particular interest because of the intimate relationship of vitamin B_6 to brain metabolism as evidenced by the occurrence of convulsive seizures during vitamin B_6 deficiency, whether induced by restricted intake of the vitamin,[8-10] by administration of analogues of the vitamin,[9, 11] or by administration of carbonyl reagents such as isonicotinyl hydrazide and related compounds.[12, 13]

We have now partially purified the brain kinase and examined some of its properties. Carbonyl reagents proved to be potent inhibitors of the enzyme, which was maximally activated only by Zn^{++}. Some details of these studies are presented herein.

Methods.—Pyridoxal phosphate formation was followed by the method of Wada *et al.*,[14] with slight modifications. This involves colorimetric determination[15] of indole production from tryptophan in the presence of apotryptophanase from *E. coli* and is a considerable simplification over previously used methods. Protein was determined by the method of Lowry *et al.*[16] and by optical density measurement at 280 mμ.

TABLE 1

PURIFICATION OF PYRIDOXAL KINASE FROM HUMAN BRAIN

Fraction	Specific Activity*	Total Activity†
(1) Brain homogenate	1.5	22,100
(2) Supernatant solution from (1)	14.2‡	47,000
(3) Dialyzed fraction of (2) soluble in 40, insoluble in 60% $(NH_4)_2SO_4$	26.7	28,000
(4) Peak cut from DEAE-cellulose column, dialyzed	250	...

* Mμmoles of pyridoxal phosphate formed per mg of protein per hr at 37°C. The incubation mixture contained 5×10^{-4} *M* pyridoxal, 5×10^{-4} *M* ATP, 1×10^{-3} *M* Zn^{++}, 0.1 *M* potassium phosphate buffer at pH 6.5, and enzyme in 2.5 ml total volume. Incubation was at 37° for 1 hr.

† From 200 gm of cerebral tissue.

‡ An independent preparation from an autopsy specimen held in the deep freeze for two years prior to fractionation showed a specific activity of 300 at this point and was used (following dialysis) in some of the experimental work.

The semicarbazone, hydrazone and α-methylphenethylhydrazone of pyridoxal were synthesized by heating approximately equimolar quantities of pyridoxal hydrochloride with the appropriate carbonyl reagent in 0.1 *M* potassium acetate buffer, pH 4.5, filtering, and washing. The 2,4-dinitrophenylhydrazone was produced from a similar reaction in ethanolic HCl. All compounds yielded the expected analysis for C, H, and N.

Pyridoxal kinase was purified as described below. All operations were carried out at 0°C. (1) Cerebral cortex was homogenized in a Tenbroeck apparatus with three to four volumes of 0.1 *M* potassium phosphate buffer, pH 6.5, and the suspension centrifuged at 18,500 × gm for 30 minutes. (2) The clear, reddish-yellow supernatant solution was decanted and brought to 40 per cent saturation with ammonium sulfate. The mixture was centrifuged at 18,500 × gm for 15 minutes, and the precipitate discarded. (3) To the supernatant solution from step 2, ammonium sulfate was added to 60 per cent saturation. The precipitate

formed contains the kinase and was collected by centrifuging for 15 minutes. It was dissolved in a small volume of distilled water and dialyzed against 0.05 *M* potassium phosphate buffer, pH 6.5, to remove ammonium sulfate. (4) The dialyzed solution was again centrifuged. The active yellowish, supernatant solution was poured over 5 to 20 times its weight (cellulose/protein) of diethylaminoethylcellulose and eluted fractionally in a linear gradient fashion with potassium phosphate buffer between 0.05 *M*, pH 6.5, and 0.2 *M*, pH 5.5. (5) Fractions containing kinase activity were pooled. The protein was precipitated at 60 per cent saturation of ammonium sulfate, collected by centrifugation, dissolved in a small volume of 0.05 *M* potassium phosphate buffer, pH 7.0, and dialyzed against this buffer.

Results.—Purification and properties of the kinase: The purification of enzyme during the fractionation is shown in Table 1. Figure 1 shows the elution pattern of the material over DEAE-cellulose. The kinase activity of brain tissue does not decrease, and may even increase, on storage in the frozen state for as long as two years. Concentrates of the kinase at stage (3), Table 1, are also quite stable to storage at deep-freeze temperatures for long periods of time, but more highly purified fractions from the cellulose column are much less stable. Unlike crude fractions of the pyridoxal kinase of liver,[7] most of the activity of the brain kinase is destroyed by heating at 55°C for 5 minutes.

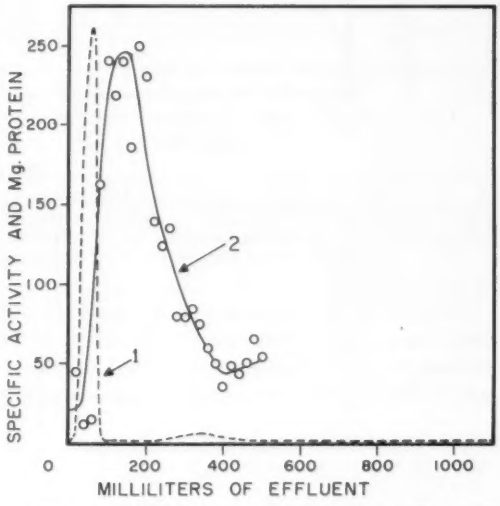

FIG. 1.—Fractionation of pyridoxal kinase from human brain on DEAE-cellulose. One gram of Fraction 3, Table 1 (sp. act. 26.7) was applied to five grams of the cellulose derivative. Assay conditions as in Table 1. Curve 1, protein; curve 2, specific activity of kinase.

The relation of pyridoxal phosphate formation to concentration of pyridoxal, adenosine triphosphate, and Zn^{++} is shown in Figure 2. The K_M value for pyridoxal was 10^{-4} to 10^{-5} *M;* the smaller value was obtained with more highly purified preparations. K_M values for ATP and for Zn^{++} were 5×10^{-5} *M* and 1×10^{-6} *M*, respectively. The pH optimum (Fig. 3) is approximately 6.5 with Zn^{++} as the activating metal ion, but is nearer 7.0 and much broader with Mg^{++} as the activating metal ion. At pH 6.5 the activity of the Zn^{++}-activated enzyme is at least twice that of the Mg^{++}-activated enzyme. Separate trials in which the concentrations of these ions were varied in systems containing optimal amounts of pyridoxal and ATP confirmed this superiority of Zn^{++}; at no concentration did Mg^{++} elicit the same high activities as did Zn^{++}. The K_M value for Mg^{++} (1×10^{-5} *M*) was approximately ten times that for Zn^{++} (1×10^{-6} *M*). From these and other experiments, near optimal conditions of assay for the kinase were established as those specified in Table 1.

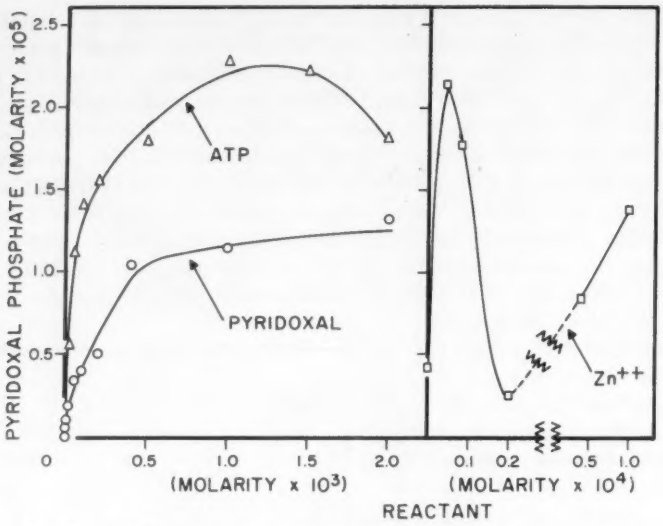

FIG. 2.—Relation of pyridoxal phosphate formation to concentration of pyridoxal, ATP and Zn^{++}. Assay conditions as in Table 1 with appropriate omission, and 0.2 mg. of kinase (sp. act. 300) per 2.5 ml. Precipitation of zinc phosphate begins at the point of decline in the activity-concentration curve.

Inhibition of the kinase by carbonyl reagents: The effects of several psychoactive drugs on activity of the kinase are shown in Figure 4. Chlorpromazine and 1-(1-phenylcyclohexyl)piperidine did not inhibit but rather stimulated the enzyme mildly, whereas the converse was observed with iproniazid phosphate. α-Methyl-

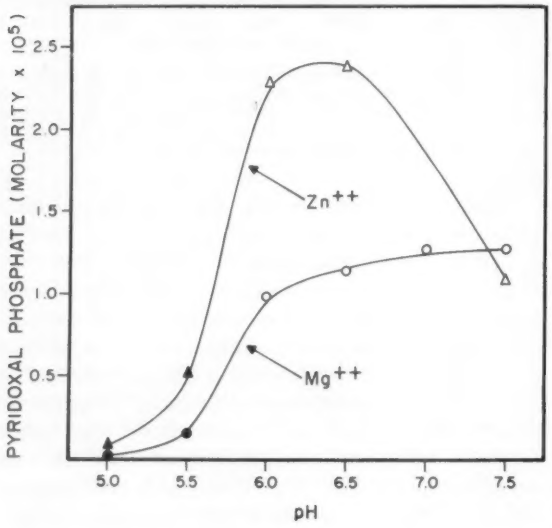

FIG. 3.—Relation of pH and activating metal ion to activity of the brain kinase. Assay conditions as in Table 1, but with Mg^{++} (1×10^{-4} *M*) substituted for Zn^{++} where indicated. 0.4 mg. of kinase (sp. act. 150 (calculated with Zn^{++} at pH 6.5)) per 2.5 ml.

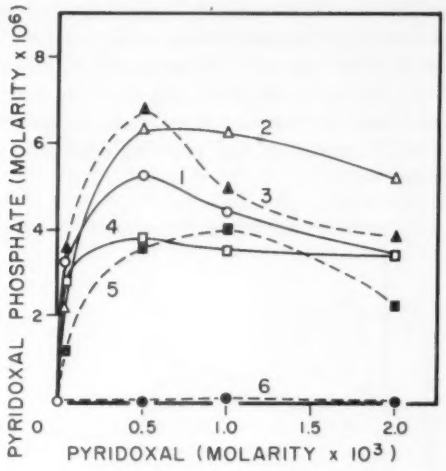

FIG. 4.—Effects of various psychoactive drugs on the activity of the brain kinase. Assay conditions as in Table 1, but with pyridoxal varied and 2×10^{-4} *M* drug added as indicated below. 0.2 mg. of kinase (sp. act. 150) per 2.5 ml. assay tube. Curve 1, control; 2, chlorpromazine; 3, 1-(1-phenylcyclohexyl)piperidine; 4, iproniazid phosphate; 5, 4-(o-(propylthio)-phenyl-1-piperazine pentanol)ethylcarbamate; 6, α-methylphenethylhydrazine.

phenethylhydrazine was an extremely potent inhibitor. The activity of the hydrazine-inhibited system was not restored by as much as a tenfold excess of pyridoxal, ATP or Mg^{++} (Fig. 5). Substrate removal or binding of essential metal ions was not, therefore, responsible for the inactivation.

That the actual inhibitor was the hydrazone, formed between pyridoxal and α-

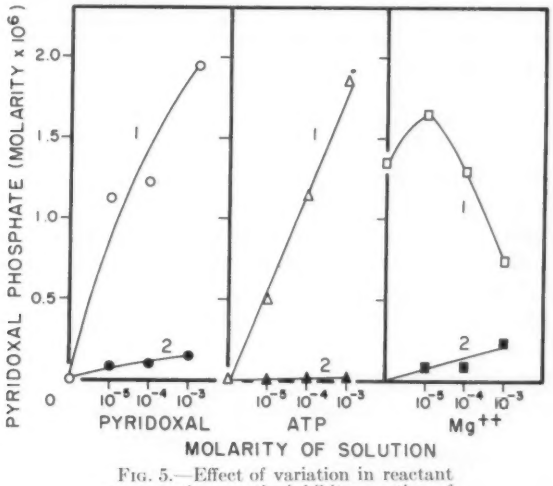

FIG. 5.—Effect of variation in reactant concentrations on the inhibitory action of α-methylphenethylhydrazine. Assay conditions as in Table 1, but with Zn^{++} replaced by Mg^{++} (1×10^{-4} *M*) and pyridoxal, ATP, or Mg^{++} varied as indicated. 0.2 mg. kinase (sp. act. 150) per 2.5 ml. assay tube. Curve 1, control; curve 2, plus 1×10^{-4} M α-methylphenethylhydrazine.

methylphenethylhydrazine, was shown by tests of the synthetic compound, which proved slightly more potent as an inhibitor than the parent hydrazine. In the presence of 5×10^{-4} *M* pyridoxal, 50 per cent inhibition of pyridoxal phosphate formation occurred with approximately 3×10^{-6} *M* hydrazone. The affinity of the kinase for this inhibitor is thus well over one hundred times that for pyridoxal itself. The severe and partially competitive nature of this inhibition is seen in Figure 6.

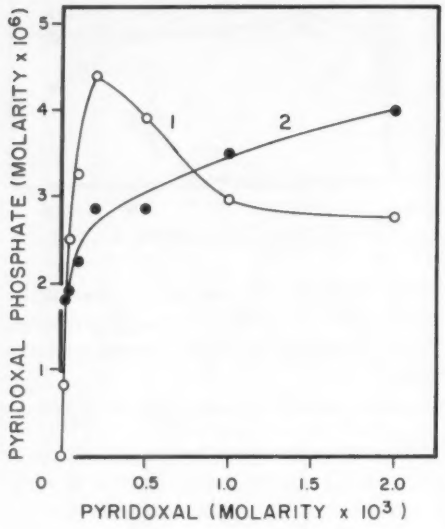

FIG. 6.—Inhibition of pyridoxal phosphate formation by low concentrations of pyridoxal α-methylphenethylhydrazone. Assay conditions as in Table 1, but with pyridoxal varied. 0.2 mg. of kinase (sp. act. 150) per 2.5 ml. assay tube. Curve 1, control; curve 2, plus 9×10^{-7} *M* hydrazone.

Similar inhibition of the purified kinase was exhibited by two other hydrazones of pyridoxal tested (Table 2) and also by pyridoxal oxime and pyridoxal semicarbazone. Suitable controls showed in all cases that the inhibition was of the kinase, and not of the tryptophanase system used for assay of pyridoxal phosphate formation. For example, pyridoxal α-methylphenethylhydrazone at concentrations that inhibited the kinase completely had no effect on the tryptophanase of *E. coli*.

Discussion.—The information available on most kinases has led to the tacit assumption that Mg^{++} is the preferred activator for all such enzymes. This assump-

TABLE 2

COMPARATIVE INHIBITORY POTENCIES OF CERTAIN CARBONYL REAGENTS AND THEIR CONDENSATION PRODUCTS WITH PYRIDOXAL FOR THE PYRIDOXAL KINASE OF HUMAN BRAIN

Compound Tested	Concentration at 50% Inhibition,* *M*
Isonicotinylhydrazide (isoniazid)	4.8×10^{-6}
Semicarbazide	1.5×10^{-5}
Pyridoxal semicarbazone	4.0×10^{-7}
Pyridoxal hydrazone	5.0×10^{-7}
Pyridoxal α-methylphenethylhydrazone	3.0×10^{-6}
Pyridoxal 2,4-dinitrophenylhydrazone	1.5×10^{-5}
Pyridoxal oxime	5.0×10^{-7}

* Testing conditions as in Table 1. 0.2 mg of kinase (sp. act. 150) per 2.5 ml assay tube.

tion appears to be based principally on extrapolation from a few carefully studied systems and data indicating that intracellular Mg^{++} is more plentiful than other divalent ions. Only infrequently have careful comparisons of the activities of different activating ions been reported; in some of these, indeed, Mn^{++} has proved as active as Mg^{++}. For the brain kinase Mg^{++}, though active, is definitely inferior as an activator to Zn^{++} under conditions optimal for enzymatic activity. Whether only one, or both of these ions functions with the kinase *in situ* is not determined by these data. Reported concentrations of both zinc[17] and magnesium[18] in brain tissue are above those required for maximum activation of the kinase in the *in vitro* system.

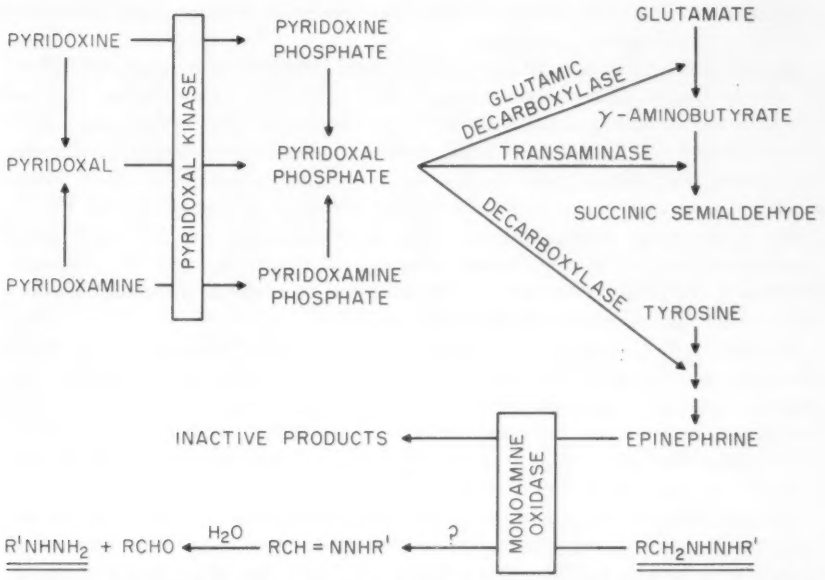

FIG. 7.—Some interrelationships among pyridoxal kinase, pyridoxal phosphate enzymes, and monoamine oxidase of importance for assessment of analeptic and convulsive actions of hydrazines and their derivatives.

Carbonyl reagents are known to inhibit most pyridoxal phosphate enzymes through formation of a coenzymatically inactive derivative with the cofactor.[19] Many of these reagents are now recognized as stimulants of the central nervous system ("psychic energizers") which, in sufficient excess, produce epileptiform seizures that may be alleviated by administration of pyridoxine.[13,20] The localization of glutamic decarboxylase in brain,[21] together with an observed reduction in levels of γ-aminobutyrate following isoniazid treatment[13] have been interpreted to mean that both the analeptic and convulsive activities of active carbonyl reagents may result from interference with production of this inhibitor of neuronal transmission by the glutamic decarboxylase.[22] The data presented here indicate strongly that this decrease in concentration of γ-aminobutyrate results from lowered pyridoxal phosphate levels as a consequence of inhibition of the pyridoxal kinase,

rather than from inhibition of the glutamic decarboxylase *per se*. A comparison of the sensitivity of kinase and decarboxylase to the hydrazine derivatives is required to settle this point;[23] however, the sensitivity of the kinase to isoniazid demonstrated here is far greater than that of the pyridoxal phosphate enzymes so far studied. Since pyridoxal kinase phosphorylates each of the three forms of vitamin B_6,[24] it is clear that pyridoxal phosphate formation from all forms of the vitamin will be inhibited by these carbonyl reagents, whichever of the possible alternate routes to the coenzyme may be followed *in vivo* (cf. Fig. 7). The sole prerequisite for such inhibition would be sufficient quantities of free pyridoxal in the tissues to form the actual inhibitor from the carbonyl reagent. That this is present is obvious from the increased excretion of vitamin B_6 that results from feeding isonicotinylhydrazide[25] and semicarbazide.[26] In the latter instance, pyridoxal semicarbazone was detected in the urine.

Several of the hydrazine derivatives are potent inhibitors of monoamine oxidase, an action thought to contribute to their analeptic action by preventing rapid removal of the sympathomimetic amines.[27] If they inhibit by serving as competitive substrates for the amine oxidase, a possible mechanism for formation of monosubstituted from disubstituted hydrazines, and hence of potent pyridoxal kinase inhibitors from compounds such as iproniazid, would be present *in vivo* (cf. Fig. 7). A few of the many possible sites at which hydrazines may interfere with known reactions of importance in brain metabolism are illustrated in Figure 7. The complexities of the possible interactions are such that more detailed speculations concerning their nature is unprofitable at this time.

Summary.—Brain tissue was found to be an unusually rich source of pyridoxal phosphokinase. A procedure for purification of this enzyme approximately 200-fold from human brain tissue is described. At its optimum pH of 6.5, the purified kinase was preferentially activated by Zn^{++} (K_M, 10^{-6} *M*) and less effectively by Mg^{++} (K_M, 10^{-5} *M*). Values of K_M for pyridoxal and adenosine triphosphate were 10^{-5} *M* and 5×10^{-5} *M*, respectively.

Because of its indirect role in formation of γ-aminobutyrate, the effects of various tranquilizers and psychic energizers on activity of the kinase were determined. Chlorpromazine and 1-(1-phenylcyclohexyl)piperidine showed a slight stimulatory action of uncertain significance; iproniazid inhibited moderately. α-Methylphenethylhydrazine and isoniazid were extremely effective inhibitors of the kinase; the actual inhibitors were found to be the corresponding hydrazones formed between these agents and pyridoxal. The hydrazone, oxime, and semicarbazone of pyridoxal are all similarly potent inhibitors, with affinities for the enzyme well over one hundred times that of the substrate, pyridoxal. Because of the low concentrations at which they exert this effect, it is considered that the physiological effects of these carbonyl reagents in bacteria and mammals result in part from their capacity to lower or eliminate production of pyridoxal phosphate by a given tissue.

* This investigation was supported in part by a grant (A-1448) from the U. S. Public Health Service. We are indebted to Parke, Davis & Co. for certain of the compounds tested.

† Post-doctoral research fellow of the U. S. Public Health Service.

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[23] Preliminary comparisons have shown that the glutamic decarboxylase of beef brain requires almost 1000-fold greater concentration of carbonyl reagents for inhibition than does the pyridoxal kinase of this tissue.

[24] Like the yeast kinase,[6] we find the brain kinase also phosphorylates pyridoxine and pyridoxamine. Affinities of these substrates for the kinase are similar to that of pyridoxal.

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INHIBITION OF IODIDE OXIDATION BY THYROXINE AND OTHER ANTIOXIDANTS

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Communicated by J. Holtfreter, August 6, 1959

Among the physicochemical properties exhibited by many bioregulators is their ability to interact with oxidation-reduction systems. A study of this function in indole-3-acetic acid,[1] serotonin, and other indoles showed them to be highly efficient antioxidants. The oxidation of iodide to iodine with hydrogen peroxide was inhibited as well by nonindolic organic compounds such as thyroxine, suggesting a regulatory role for thyroid hormones in a primary step[2] in their own formation.

This paper deals with the antioxidant activities of selected organic substances and some physical and biological implications of this property.

Methods.—The iodide oxidation system has been employed in two modifications.

First, as previously described,[3] in metal-ion insensitive noncatalytic form, and second, as a peroxidase-catalyzed reaction. Reaction conditions were KI, 2–10 m moles L^{-1}; H_2O_2, one-tenth the KI concentration; Buffer, $M/15$ phosphate, pH 6.6; enzyme, crystalline horseradish peroxidase, 0.002 μmoles L^{-1}. When pyrogallol replaced KI as substrate, it was used at 5 μmoles L^{-1} and H_2O_2 at 10 μmole L^{-1}. Reactions were carried out at 25°C.

Iodine formed by iodide oxidation was determined photometrically at 360 mμ as KI_3. Readings were taken for 10 min at 1 min intervals to establish the linearity of the reaction. Oxidation of pyrogallol to purpurogallin was followed photometrically at 425 mμ.

Experimental Observations.—Characteristics of thyroxine inhibition: Representative data show that thyroxine inhibits iodide oxidation at low concentrations with or without catalyst (Table 1). The faster enzymatic reaction is also appreciably more sensitive to thyroxine.

TABLE 1

EFFECT OF THYROXINE ON IODIDE OXIDATION

Substrate, m moles L^{-1}	H_2O_2, m moles L^{-1}	Thyroxine, μmoles L^{-1}	Product, μmoles $L^{-1}min^{-1}$	Inhibition %
		A. Nonenzymic		
2.0	0.2	0	0.09	
		10	0.03	67
5.0	0.5	0	0.47	
		10	0.22	53
10.0	1.0	0	4.00	
		10	2.25	44
		B. Peroxidase 0.002 μmoles L^{-1}		
5.0	0.5	0	2.48	
		2	1.18	52
		10	0.84	66
5.0 (Pyrogallol)	10.0	0	275 (Purpurogallin)	
		10	281	..
		100	303	..

Of the noncatalytic and catalytic oxidations the concentrations for 50 per cent inhibition are 10 and 2 μmole L^{-1} respectively. In the enzymic system, thyroxine is a minor component relative to all reactants save peroxidase. To distinguish between inhibition of reaction and of enzyme, pyrogallol was substituted for iodide (Table 1-B). It is evident that thyroxine does not act as a general peroxidase poison.

When measurements were extended to longer reaction times, the amounts of I_2 formed in control and inhibited systems could be compared with the quantity of thyroxine present. From such comparisons, it was calculated that each mole of thyroxine suppresses the appearance of approximately 7 moles of I_2. This ratio holds for periods of 2 hrs. Inhibitory efficiency declines thereafter, but inhibition is still manifest after reactions of some 4 or 5 hr. duration.

The behavior of thyroxine as a monophenolic reducing agent can be described by the half-reaction

$$R - C_6H_4OH \rightleftharpoons R - C_6H_4O\cdot + H^+ + e^-$$

In a simple oxidation-reduction reaction, one mole of inhibitor would be consumed for each 0.5 moles of I_2 reduced. Accordingly, our observed ratio of 7:1

is approximately 14-fold greater than that which could be accounted for if a simple, noncatalytic effect were assumed.

The relatively low catalytic efficiency observed and the eventual decline in activity probably result from attack by peroxidase upon the phenolic group of thyroxine in a medium of high H_2O_2 concentration.

Comparison of thyroxine and its relatives: In spite of its reducing activity as a phenol, tyrosine shows little inhibitory activity (Table 2). Iodination elevates activity somewhat, but does not have the effect obtained by coupling aromatic nuclei via an ether linkage (thyronine). Even the parent compound of the thyronine series, diphenylether possesses weak inhibitory activity (40 percent at 200 μmole L^{-1}).

TABLE 2

INHIBITION OF ENZYMATIC IODIDE OXIDATION BY THYROXINE AND RELATED COMPOUNDS

Compound	Inhibition at 2 μmole L^{-1}, %
dl-Tyrosine	10
3,5-Diiodotyrosine	18
Thyronine	40
3,5-Diiodothyronine	40
3,3′,5-Triiodothyronine	46
3,3′,5,5′-Tetraiodothyronine	55

Other iodide antioxidants: A number of heterocyclic compounds of physiological interest possess inhibitory activity. Some of these have been studied in relation to plant-growth regulatory activity and have been[3] or will be reported on separately. Active compounds of especial interest here include: indole, indoline, pyrrolidine, pyrrole, bile pigments, serotonin, and reserpine. This list covers structures found in alkaloids, neurohormones, and porphyrins. An additional hormonal substance of high inhibitory activity is epinephrine.

Oxidation of the indole nucleus (oxindole, insatin) destroys antioxidant activity, as does replacement of the heteroatom with —CH_2—; substitution of —S— or —O— for —NH— reduces activity, but does not remove it completely.

Discussion and Conclusions.—Consideration of inhibitory mechanisms: The data presented here, although preliminary, suggest the following characteristics: (1) Inhibition is independent of the metal-complexing properties of thyroxine[4] (metal-ion insensitive iodide system); (2) The inhibitory effect of thyroxine in enzymic oxidation involves the reaction itself not the enzyme; (3) Inhibitory efficiency is high enough to suggest that thyroxine (and other compounds) participate reversibly; (4) Current findings together with earlier ones show high inhibitory activity to be dependent upon a common factor, other than simple reducing properties, in a diverse array of organic molecules; (5) Relative to thyroxine itself, both iodination and the ether coupling between phenyl groups may contribute to high inhibitory efficiency.

The foregoing points support a suggested role for thyroxine in electron transport.[4] This suggestion, based on spectroscopic behavior of the thyronines and the present data call attention to the probable importance of electron availability in the antioxidant function.

The existence of a combined system of nonbonding and π-orbital electrons may well be the common property referred to above. Additional support for this

hypothesis comes from the demonstration that the activity of arylamines as antioxidants is independent of N-H scission, but favored by electron-supplying groups,[5] and the molecular orbital calculations of the Pullmans[6] which show that aromatic amino acids are moderately good π-orbital electron donors. The loss in activity among indoles referred to briefly—by introduction of ring carbonyl groups or replacement of N with C—seems to offer further support.

Further evidence of an entirely distinct character has been provided by Szent-Györgyi in his novel quantum mechanical treatment of bioenergetic phenomena.[7] This work emphasizes the importance of the nonbonding electrons of N, S, and O in the phosphorescence- and fluorescence-quenching properties of their compounds, and specifically calls attention to the introduction of iodine into extensively resonating aromatic structures—i.e., thyroxine—as a case in point. Examination of the most likely mechanism of iodide oxidation[8] suggests two sites for antioxidant action: (1) interference in the initial nucleophilic attack by I^- upon $H_3O_2^+$; (2) direct attack by antioxidants upon HOI, the most highly oxidized intermediate. It is hoped that work now in progress will distinguish between these alternatives.

Physiological implications: In the preceding discussion, iodide oxidation has been treated as a purely chemical phenomenon without regard to its biochemical importance. It is, however, the first event of a chain leading to release of thyroxine into general circulation. It is suggested, therefore, that thyroxine and related compounds may comprise a feedback loop, linking hormone output to oxidative formation of precursor organoiodine compounds. The present work serves only as the starting point for such a physiological model, but the possibility that such a self-regulatory system exists seems to merit further exploration.

Summary.—The oxidations of iodide H_2O_2 with or without catalyst (peroxidase) is inhibited by a number of organic compounds including selected heterocyclic compounds, thyroxine, and its relatives. Their high inhibitory efficiency suggests that these compounds function as antioxidants.

The evidence presented together with independent observations support a hypothesis that antioxidant activity derives from the donor properties of systems with nonbonding and/or π-orbital electrons. A consideration of the inhibition of iodide oxidation itself leads to the proposal that thyroid hormone output may be coupled to formation of its organoiodine precursors as a regulatory feedback system.

Thanks are due Dr. V. Schomaker of this Institute for the discussions and advice which helped to direct our antioxidant research toward its present orientation.

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ANALYSIS OF CONDUCTANCE DATA FOR 1-1 IONOPHORES

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The reduction of conductance data (equivalent conductances at a series of properly chosen concentrations for a given 1-1 electrolyte in a given solvent at a fixed temperature) to molecular parameters characteristic of solute and solvent is simple in two general cases:[1] (1) that of negligible association to ion pairs, and (2) that of appreciable association to pairs but negligible association to clusters[2] containing more than two ions. In Case 1, the Fuoss-Onsager equation[3]

$$\Lambda = \Lambda_0 - Sc^{1/2} + Ec \log c + Jc \tag{1}$$

can readily be reduced to a linear function

$$\Lambda' \equiv \Lambda + Sc^{1/2} - Ec \log c = \Lambda_0 + Jc \tag{2}$$

because S and E are theoretically predictable constants (given an easily obtainable[4] first approximation for Λ_0) and a simple extrapolation then evaluates Λ_0, the limiting conductance, and J, which is an explicit function of a, the center-to-center distance of anion and cation at contact ("ion size") and from which a is evaluated. In Case 2, corresponding to association constants in the approximate range $2 < \log K_A < 4$, the Fuoss-Kraus[5] equation

$$\Lambda = \gamma(\Lambda_0 - Sc^{1/2}\gamma^{1/2}) \tag{3}$$

is an adequate approximation, because the (opposing) terms in J and E due to long range interionic effects become negligible,[6] and (3) can be reduced to a linear function,[7] extrapolation of which then evaluates Λ_0 and K_A. In order to determine a in this case, data in a series of mixtures of a polar and a nonpolar liquid are needed; a is then derivable from the slope of the corresponding $\log K_A - D^{-1}$ plot.[8, 9]

The conductance function[10] for which (1) and (3) are, respectively, the limiting forms for small and large values of K_A is

$$\Lambda_\eta = \Lambda_0 - Sc^{1/2}\gamma^{1/2} + Ec\gamma \log c\gamma + Jc\gamma - K_A c\gamma f^2 \Lambda_\eta \tag{4}$$

which involves three arbitrary constants, Λ_0, J and K_A. When data of very high precision such as those of Kraus[11] and co-workers are available, the Fuoss y-x method[10] serves to analyze the data in the transition region between Cases 1 and 2. Briefly described, this method consists in using trial values of Λ_0 in order to find that value which linearizes a rearranged form of (4): K_A and J are then determined from slope and intercept of the final plot. As implied above, this method is extremely sensitive to experimental errors, because $J(a)$ appears as a small difference between two large quantities. Values of a so obtained frequently show some systematic trend with solvent composition, which may well be merely the consequence of small errors in conductance, dielectric constant or viscosity, rather than a variation of eventual theoretical significance. If, however, a-values were available from some other source, then (4) could be reduced to a two-parameter equation and

analysis would again be simple. It is the purpose of this paper to present such an analytical method, using as examples data for tetramethyl- and tetraethyl-ammonium picrates in methanol-butanol mixtures.[12]

A recent semiempirical treatment[13] of the dependence of the Walden product on solvent composition shows that a hydrodynamic radius, characteristic of a given ion in a given pair of solvents, can be obtained by extrapolating the apparent Stokes radius to infinite dielectric constant. In the few cases for which data were available, the sum $a_\Lambda = (R_\infty^+ + R_\infty^-)$ of the radii of the cation and anion so determined were in good agreement with a_K and a_J, the ion sizes obtained, respectively, from the corresponding log $K_A - D^{-1}$ plots and from $J(a)$. As a working hypothesis, we shall make the assumption that a_Λ may be used to compute J; then (4) reduces to

$$\Lambda_K \equiv \Lambda_\eta + Sc^{1/2}\gamma^{1/2} - Ec\gamma \log c\gamma - J(a_\Lambda)c\gamma \tag{5}$$

$$= \Lambda_0 - K_A(c\gamma f^2\Lambda_\eta) \tag{6}$$

and it is immediately obvious that Λ_K so defined is a linear function of the variable

$$Z = c\gamma f^2\Lambda_\eta \tag{7}$$

At first glance, this procedure appears to be one of arguing in a circle; extrapolation of (6) evaluates K_A and Λ_0 but one must have values of Λ_0 in order to compute S, E and $J(a_\Lambda)$ and then Λ_K, which is extrapolated as a function of Z in order to obtain Λ_0. Actually, the process in practice is one of successive approximations. The J-term is small, and furthermore only the numerical difference between it and

TABLE 1

PROPERTIES OF METHANOL-BUTANOL MIXTURES

(D = dielectric constant, η = viscosity and ρ = density)

No.	Wt. % BuOH	D	100 η	ρ	Λ_0 (prelim.)
		Me_4NPi systems			
1	0.00	32.66	0.5525	0.7867	115.95
2	9.57	31.10	0.6000	0.7882	105.33
3	19.72	29.47	0.6650	0.7898	94.20
4	30.15	27.78	0.7500	0.7917	83.00
5	39.80	26.33	0.8415	0.7935	73.35
6	51.41	24.35	0.9785	0.7957	62.00
		Et_4NPi systems			
7	0.00	32.66	0.5525	0.7867	107.65
8	15.07	30.20	0.6325	0.7891	93.01
9	29.83	27.85	0.7400	0.7916	79.00
10	41.20	26.00	0.8550	0.7937	68.43
11	55.09	23.75	1.0375	0.7964	56.20

the E-term occurs in Λ_K. Neglecting both the J and E terms permits a preliminary extrapolation of the data by means of (3) and the values of Λ_0 so obtained are then used to obtain a_Λ, as previously described.[13] (If necessary, the computation may be recycled, of course.) The a_Λ values have one marked advantage over the a_J values obtained by the y-x method: they depend on the *total* observed conductance, while the latter depend essentially on the *second derivative* of the Λ-$c^{1/2}$ function. Hence data of less than ideal precision may be analyzed by the Λ_K-Z method, which avoids the extremely sensitive y-x plot.

We now consider several examples of the above method. The conductance of

tetramethylammonium picrate (Me_4NPi) and of tetraethylammonium picrate (Et_4NPi) has recently[12] been measured at 25° in a series of methanol-butanol (MeOH-BuOH) mixtures covering the approximate range 24–33 in dielectric constant, which is precisely the transition range from Case 1 to Case 2 for these systems. The properties of the solvents are summarized in Table 1, together with the preliminary values of Λ_0. Using the following values—λ_0 (Br^- in MeOH) = 56.55,[14] Λ_0 (Bu_4NBr in MeOH) = 96.15,[15] Λ_0 (Bu_4NPi in MeOH) = 86.10,[16] and Λ_0(Me_4NPi in MeOH) = 115.94[16]—we obtain for the limiting transport number of the Me_4N^+ ion of Me_4NPi in MeOH the value $n^+ = 0.600$. Assuming n^+ to be independent of solvent composition, limiting single ion conductances were computed

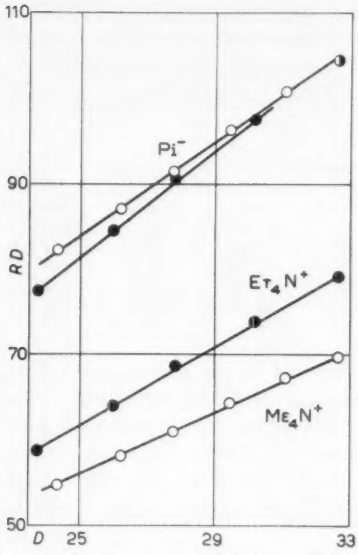

FIG. 1.—Evaluation of ionic radii by means of (9). Open circles, Me_4NPi; solid circles Et_4NPi.

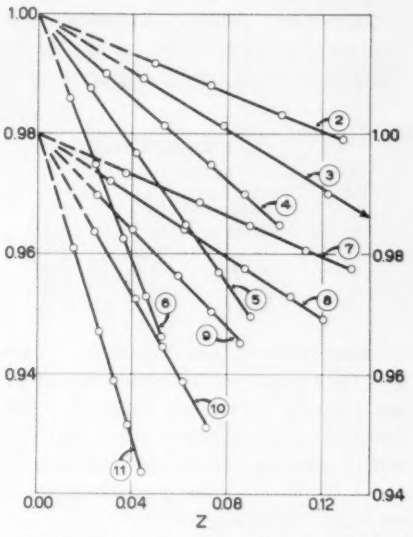

FIG. 2.—Test of equation (6); see Table 1 for code. Ordinates, Me_4NPi, left scale; Et_4NPi, right scale.

for Me_4N^+ and Pi^- in solvents 1–6; then Stokes radii were computed by means of the equation

$$R^* = Fe/1{,}800\ \pi\eta\lambda_0^* \tag{8}$$

Similarly, for Et_4NPi in MeOH, we find $n^+ = 0.569$ and the corresponding Stokes radii were calculated for the salt. Figure 1 shows plots of R^*D against D; they are linear, and are reproduced by the equations[13]

$$RD = s + R_\infty D \tag{9}$$

where the constants (distances in Å) have the following values: $R_\infty{}^+ = 1.81$, $s^+ = 10.5$, $R_\infty{}^- = 2.72$, $s^- = 16.0$ for Me_4NPi and $R_\infty{}^+ = 2.30$, $s^+ = 4.14$, $R_\infty{}^- = 3.04$, $s^- = 4.86$ for Et_4NPi. Ideally, of course, the value of R_∞ for the picrate ion should be the same from both salts; as seen in Figure 1, there is a small

difference (2.72 vs. 3.04) in the slopes; the lack of perfect coincidence is a consequence of the incomplete adequacy of (9). Nevertheless, the sums $a_\Lambda(Me_4NPi) = 4.53$ and $a_\Lambda(Et_4NPi) = 5.34$ are in the correct sequence, with a reasonable difference (0.8 Å) to correspond to the extra methylene in the ethyl groups over the methyl groups, and still more important, are in fair agreement with the dimensions expected from molecular models. They therefore seem to be adequate for use in computing the correction terms $J(a)$ needed for calculation of Λ_K according to (5).

Figure 2 shows the normalized $\Lambda_K - Z$ plots for the systems 2–11 of Table 1 (code numbers in the first column). In order to simplify the figure, the original $\Lambda_K - Z$ curves were normalized by dividing the ordinate scale by the appropriate Λ_0 values, so that all the curves terminate at unity at zero concentration. It will be noted that the plots are all linear, well within the experimental error of about ± 0.02 Λ-units. The slopes increase regularly as the dielectric constant of the solvent medium decreases, corresponding to increased association with increasing electrostatic potential energy of pairs in contact. In Table 2 is given a summary of the

TABLE 2

LIMITING CONDUCTANCES AND ASSOCIATION CONSTANTS

Me₄N Pi				Et₄N Pi			
No.	Λ_0	K_A	$10^3 c_m$	No.	Λ_0	K_A	$10^3 c_m$
1	116.20	18.7	2.7	7	107.69	18.4	2.0
2	105.38	17.2	2.1	8	93.00	24.0	2.4
3	94.22	22.8	3.7	9	78.95	32.5	2.1
4	83.00	28.3	2.5	10	68.50	46.3	2.2
5	73.33	41.2	2.8	11	56.20	71.5	1.7
6	62.07	64.0	1.9				

constants derived from Figure 2. The columns labelled $10^3\ c_m$ give in round numbers the highest concentration measured in a given system and correspond to the last points to the right in Figure 2. This figure thus serves to give some feeling for the order of magnitude of the change in conductance as a function of concentration and of association constant; for example, in system 11 at $c \approx 1.7 \times 10^{-3}$, with $K_A \approx 70$, the decrease in conductance due to pair formation amounts to about six per cent.

In Figure 3 is shown the dependence of association on dielectric constant; the log $K_A - D^{-1}$ plots are linear. If solvent-solute interaction terms[17] in K_A are disregarded, the association constant is given[9] by

$$K_A = K_\infty \exp(e^2/aDkT) \tag{10}$$

where K_∞ is the association constant describing chance collisions between uncharged particles; it can be shown[9] that

$$K_\infty = 4\pi Na^3/3{,}000; \tag{11}$$

for $\delta = 5$, $K_\infty = 0.32$. From Figure 3, we find $\delta_K = 3.85$ for Me_4NPi and $\delta_K = 4.74$ for Et_4NPi; the difference is about equal to the difference found above for the a_Λ values, but the absolute values are somewhat smaller (by 0.68 and 0.60 Å, respectively). The smaller a_K value can be accounted for by assuming that the field of the picrate ion consists of the superposition of the fields of a free charge and of a dipole.[18] For K_∞, the values are respectively 0.162 and 0.485 for Me_4NPi and Et_4NPi; the cube root of the ratio is 1.44 which is in fair agreement with

4.74/3.85 = 1.23, the ratio of the corresponding a_K values, as required by (11). The discrepancy can of course be due either to inadequacies of the sphere-in-continuum model, or to different ion-solvent interaction terms in K_A for the two cations.

The data cited also permit discussion of another point in the transition from Case 1 to Case 2. First, a reference conductance[19] may be defined:

$$L = \Lambda_0 - Sc^{1/2} + Ec \log c \tag{12}$$

It represents the limiting conductance, corrected for the (first approximation to the) theoretically predictable variation of conductance due to that part of the long range interaction which is independent of ion size. Then from (5), we find

$$\Lambda = L + (J - K_A\Lambda_0)c + O(c^{3/2}) \tag{13}$$

and the observed conductance curve will lie above or below the reference curve (13) according to whether J is greater or smaller than $K_A\Lambda_0$. Both J and K_A

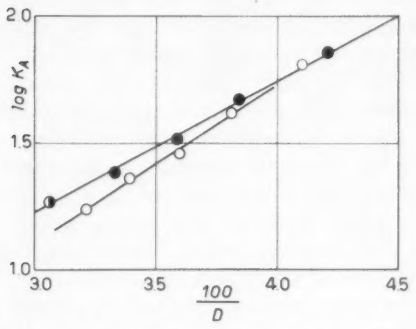

FIG. 3.—Dependence of association constant on dielectric constant. Open circles, Me_4NPi; solid circles, Et_4NPi.

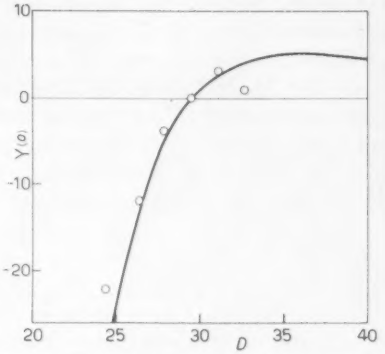

FIG. 4.—Test of equation (17) for Me_4NPi in MeOH-BuOH mixtures.

increase with decreasing dielectric constant but in quite different ways, as will be shown shortly. The coefficient of c in (13) is the intercept $y(0)$ at zero concentration in the y-x function;[10] in order to normalize the curves and eliminate the primary effects of viscosity, we define

$$Y = y/\Lambda_0 \tag{14}$$

Then

$$Y(0) = J/\Lambda_0 - K_A \tag{15}$$

Explicitly, $J = \sigma_1\Lambda_0 + \sigma_2$, but the second term σ_2 is smaller than the first;[20] for the following argument, we shall neglect it. Then using (15) and the identity

$$\kappa^2a^2/c = \pi Nba^3/125 \tag{16}$$

it can be shown by simple algebraic manipulation that

$$Y(0) \approx (\pi Na^3/1{,}500)f(b) \tag{17}$$

where

$$f(b) = (1.15b^3 \log 10b + 2b^2 + 2b - 1) - 2e^b \qquad (18)$$

For small (and physically absurd) values of $b = e^2/aDkT$, $f(b)$ approaches minus infinity *via* $\log b$; for the approximate range $1 < b < 5$, $f(b)$ is positive, and for $b > 5$, $f(b)$ again becomes negative as the exponential association term e^b dominates the positive term in parentheses in (18). In Figure 4 is shown a plot of $Y(0)$ as a function of dielectric constant, using $\mathring{a} = 3.85$; the points are the experimental values for Me_4NPi. Considering the approximations made, the agreement is excellent. The observed conductance curve can lie below the Onsager tangent, even in cases of negligible association, provided $Ec \log c$ is greater than Jc in the working range of concentration; the transition from negligible to appreciable association corresponds to the observed conductance curve moving from above L to below it with decreasing dielectric constant.

The fact that K_∞ and a_K (as well as ion-solvent interaction terms in K_A) are specifically characteristic of the ions involved explains the curious reversal noted for Me_4NPi and Et_4NPi. In ethylene dichloride[21] ($D = 10.23$), pyridine[22] ($D = 12.01$) and acetone[23] ($D = 20.47$), the association constant for Me_4NPi is larger than that for Et_4NPi, as might be expected as a consequence of the smaller size of the Me_4N^+ ions. But Me_4NPi shows negligible association in water[24] while Et_4NPi shows a slight association[18] ($K_A \approx 0.8$) in this solvent, and also in methanol, the ethyl salt is *more* associated than the methyl salt (cf. Fig. 3), despite its larger size. Since $K_\infty \sim a^3$ while e^b varies exponentially with $1/a$, we would expect K_A to be larger for the *larger* ion at high dielectric constants where e^b is dominated by a^3, while for lower dielectric constants K_A should be larger (as indeed it is) for the smaller ion, where now the exponential term in K_A has assumed control. Stated graphically, the slope of the $\log K_A - D^{-1}$ plot increases as a decreases, while the location of the intercept at $D = \infty$ decreases as a decreases; hence a crossover on this plot for two different salts is in general to be expected.

[1] Symbols used in the following discussion are defined in *La Conducibilità Elettrolitica*, R. M. Fuoss and F. Accascina, Edizione dell'Ateneo, Rome, 1959 (American Edition, Interscience Publishers, New York, 1959).

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*SOME ASPECTS OF PHOSPHATE CHEMISTRY**

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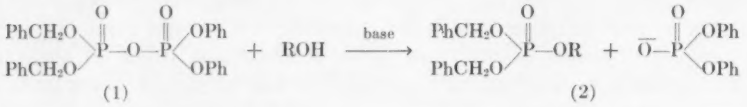
During the past fifteen to twenty years a very large amount of work has been done on organic phosphates and polyphosphates. Some have been developed for industrial or military purposes, e.g., in the insecticide and war-gas fields, but a considerable proportion has been a consequence of systematic attack on nucleotide chemistry, mainly in Cambridge and in other schools developed by former members of the Cambridge laboratory. In addition, biochemical studies on phosphates have increased greatly in recent years and, somewhat belatedly, physicochemical investigations are now being undertaken. Although much more physicochemical work will be needed to provide accurate quantitative data, it is now possible to make a number of generalisations on the evidence to hand, and to make a variety of predictions which are, I believe, of general interest, not only from a chemical, but also from a biological standpoint. For much that has hitherto seemed obscure in biochemical processes involving phosphate and polyphosphate esters is now recognised as being merely a manifestation of properties which can be demonstrated in the laboratory and it is certain that as we extend our purely chemical knowledge of the phosphates the remaining apparent anomalies will also disappear. In this paper I intend to consider firstly the methods by which phosphorylation may be brought about (i.e., formation of esters, amides, and anhydrides of phosphoric acid derivatives), and secondly the use of phosphoric acid derivatives as alkylating agents capable of forming *inter alia* carbon-carbon linkages.

Phosphorylation.—If we omit from consideration the long established and frequently unsatisfactory method of phosphorylating alcohols by direct reaction with phosphoryl chloride in presence of a base, modern methods of phosphorylation fall into two broad groups—(1) those which yield as initial products triesters of phosphoric acid (or, in the case of anhydrides, fully esterified polyphosphates) and in which the phosphorylating entity derives from a diester of phosphoric acid, and (2)

those based on agents prepared from monoesters of phosphoric acid and yielding as initial products diesters of phosphoric acid. Although it has been customary in the past to regard these two groups as similar, it may well be that they are often quite different from one another, and, as will be shown later, a distinction between them helps to clarify a number of otherwise puzzling observations.

Group 1 Methods: The first major class of phosphorylation procedures falling into this group can be loosely described as the *anhydride class*, i.e., that in which a tetraester of pyrophosphoric acid, the mixed anhydride of a phosphodiester with a stronger acid, or a diester of, e.g., phosphorochloridic acid is employed as phosphorylating agent, usually in presence of a base. In all such cases there are two possible mechanisms to consider: (*a*) the anhydride may first ionise before it phosphorylates or (*b*) the intact molecule may be the active reagent. This is, of course, equally true of carboxylic acid anhydrides when used as acylating agents, but, whereas in, say, the mixed anhydride of trifluoracetic acid and acetic acid the two mechanisms lead to different results, in the case of the phosphoric anhydrides both give effectively the same result. Under normal conditions of phosphorylation in presence of a base a mixed pyrophosphate (1) appears always to yield predominantly, if not exclusively, a phosphate (2) derived from the weaker of the two acids composing the pyrophosphate. Similarly, a mixed anhydride of a phosphodiester and a sulphonic acid or trifluoroacetic acid will be a phosphorylating agent while a corresponding mixed anhydride with, e.g., acetic acid will be an acylating rather than a phosphorylating agent.



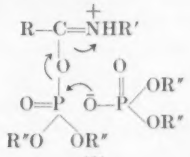
(1) (2)

In the event of ionisation (or effective ionisation) of such an anhydride as (1) it is clear that the phosphorylium cation (the phosphorylating entity) would derive from the weaker of the two acids, the anhydride oxygen remaining attached to the phosphorus of the stronger acid as part of its anion. In the intact anhydride molecule, however, both the phosphorus atoms carry a measure of positive charge, that belonging to the stronger acid being the more positive of the two. An approaching nucleophile might therefore be expected to attack both phosphorus atoms. But, since the P=O group has no additive properties in the phosphates, any such attack can result in attachment of the nucleophile only if there is simultaneous expulsion of a group on the other side of the phosphorus atom. Hence, although a nucleophile can, and doubtless does, approach both phosphorus atoms, the overwhelming proportion of successful attacks will be those which cause simultaneous expulsion of the most stable anion, i.e., that of the stronger acid, and hence for the most part phosphorylation by the weaker of the two acids is observed.[1] In sharp contrast mixed anhydrides of carboxylic acids when reacting as intact molecules normally acylate with the stronger of the two acids forming the anhydride, since the C=O group has additive properties and an approaching nucleophile will therefore tend to attach itself to the more positive of the two carbonyl carbons present, i.e., that belonging to the stronger acid.

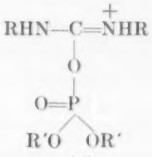
This behaviour of the phosphoric anhydrides (which probably applies also to

those of other strong inorganic oxy-acids) is well illustrated in the so-called exchange reaction of pyrophosphates[2, 3] wherein if a pyrophosphate A-B derived from two different phosphodiesters A and B is brought into contact with a salt of a third phosphodiester, C, then, provided the order of stability of their ions is B> C> A, we will observe the reaction A − B + C → A − C + B. This reaction, in a somewhat modified form to be discussed later, is probably the basis of many biological synthetic processes involving polyphosphates.

In the above methods of phosphorylation using acid anhydrides the driving force can be regarded as the expulsion of a stable anion. But there is a second type of method known in which a phosphorylating reagent is produced by protonation, usually of a compound containing the grouping $-N=C-OP(O)(OR)_2$, i.e., an imidoyl phosphate. Reagents of this nature include the simple imidoyl phosphates and the hypothetical intermediates in the reaction of phosphates with carbodiimides,[5] ketenimides, and cyanamide derivatives.[6] A typical example of such a reagent is the protonated imidoyl phosphate (3) which reacts readily with a phosphodiester anion as shown to yield a pyrophosphate. The reactive intermediate in the reaction between a carbodiimide and a phosphodiester is believed to have structure (4). In each case the electronic displacements indicated lead to ready attack on phosphorus by an anion with expulsion of the nitrogenous esterifying group as an amide.



(3)

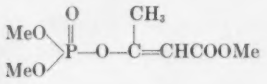


(4)

It is of interest to note that, using diesters of phosphoric acid as starting materials in these imidoyl phosphate methods, pyrophosphate preparation is very much favoured, whereas esterification of alcohols to yield phosphotriesters is usually difficult. This would suggest that actual fission of intermediates like (3) to give phosphorylium cations does not occur; this would be in line with common laboratory experience that compounds such as tetrabenzyl and tetraethyl pyrophosphate which probably react as intact molecules are very indifferent phosphorylating agents for weak nucleophiles like alcohols, whereas reagents like dibenzyl phosphorochloridate are effective for this purpose.

Since imidoyl phosphates can be rendered effective phosphorylating agents by addition of a proton, it should be possible to develop phosphorylating agents from other types of phosphate esters by electron withdrawal. Such a process would be, of course, an oxidative phosphorylation and its reality has been demonstrated in a number of laboratory examples. Oxidative phosphorylation has not yet been exhaustively studied from a preparative standpoint, but its general scope and limitations should be like those of the imidoyl phosphate procedures. The first group of compounds to be considered in this connection is that of the simple enol-phosphates. It has been shown that the enol-phosphate (5) used as a systemic insecticide under the name *phosdrin*, like dialkyl esters of other enol-phosphates of

simple α-ketocarboxylic acids, is very resistant to hydrolysis at pH 9; but addition of an oxidising agent like bromine or hypobromite to an aqueous solution at this pH causes very rapid breakdown with formation of dimethyl phosphate; presumably the breakdown is a consequence of a process of the type pictured in (6).[7] It should be noted in passing that while enol-phosphates such as (5) may phosphorylate only under oxidising conditions, it is possible that, given appropriate structural features ensuring a very powerful electron-withdrawal from the P-O linkage, some enol-phosphates might phosphorylate directly; such appears to be the case with enol-phosphates of malonic ester derivatives.[8]



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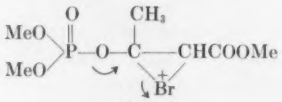

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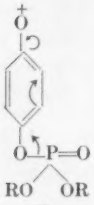

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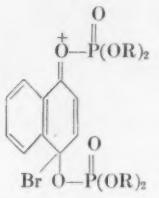


(8)

Even more striking in their behaviour as phosphorylating agents under oxidising conditions are the quinol monophosphates and diphosphates, as shown by Clark, Kirby, and Todd.[9] Although quite resistant to alkaline hydrolysis in the absence of air, addition of an oxidising agent such as bromine or a ceric salt to their aqueous solutions causes immediate precipitation of quinone; if the experiment is carried out in presence of phosphate ions pyrophosphate is produced. This oxidative phosphorylation may proceed *via* an intermediate of type (7) in the case of quinol monophosphates. With quinol diphosphates (8) may represent the initial product of attack by bromine on 2-methyl-1:4-naphthaquinol diphosphate and in this case of necessity two phosphorylating entities are produced in the oxidation as well as bromide ion. Wieland[10] has also demonstrated oxidative phosphorylation using quinol phosphates and iodine and has shown a similar phosphorylation with esters of thiolphosphoric acid and iodine (where oxidation at the sulphur atom initiates the reaction).

The very rapid oxidative phosphorylation observed with 2-methyl-1:4-naphthaquinol diphosphate is particularly striking since this substance, which is commercially available as a vitamin K substitute under the name Synkavit and is a radiosensitizer and a powerful inhibitor of mitosis in chick heart fibroblasts, undergoes *in vivo* a rapid dephosphorylation which may be oxidative in type. Moreover, the participation of vitamin K in phosphorylation processes has been frequently postulated. The general thesis that quinol phosphates may play an important role in biological oxidative phosphorylation would also accord with the widespread oc-

currence of quinone derivatives such as the ubiquinones and coenzyme Q in biological systems.[11]

Although the quinol phosphate reactions seem very well suited to biological oxidative phosphorylation the problem of quinol phosphate formation from inorganic phosphate remains to be answered if they do function in this way. The possibility that valency changes in heavy metals may be involved in making phosphate ion into a phosphorylating entity—either as a radical or as metaphosphate—is being studied in Cambridge, both as regards direct production of pyrophosphate from phosphate and the reductive phosphorylation of quinones.

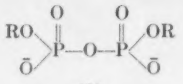
So far we have considered three types of phosphorylation processes—the anhydride type, the imidoyl phosphate type and the oxidative type. Where the phosphoric acid residues in the reagents are fully esterified, i.e., when used to form triesters (or fully esterified pyrophosphates), it is clear that they are fundamentally similar and that where the agent reacts as an intact molecule it is primarily useful in reaction with certain anions (i.e., in pyrophosphate formation) or with other strong nucleophiles such as amines. To be of any practical value in phosphorylating alcohols, it would seem that there must be incipient ionisation of the reagent and that the diesterified phosphorylium cation is the operative agent.

In my view, however, the weight of available evidence suggests that in those cases where the phosphorylating agent derives from a monoester of phosphoric acid, i.e., where the objective is production of a diester of phosphoric acid (or di- and tri-esters of pyrophosphoric acid), the mechanism is different in so far as the phosphorylating entity is normally a monomeric derivative of metaphosphoric acid. (It should be mentioned here that such reagents as phenyl phosphorodichloridate ($PhOPOCl_2$) do not belong to this type; they are best regarded as diesters of phosphorochloridic acid in which one ester group has been replaced by chlorine.) These methods are best discussed together as the second group of phosphorylation procedures.

Group 2 Methods: P_1P_2-Diesters of pyrophosphoric acid (9) cannot be used as phosphorylating agents in the laboratory, presumably because approach of a nucleophile to either phosphorus atom is prevented by the negative charge associated with each of them in the anion. Triesters (10) so far studied are also of little practical value; reaction at the monoesterified phosphorus is inhibited, although there is some evidence that exchange reactions may take place involving the fully esterified phosphoric acid residue in them.[12] Insufficient work has been done on examples of type (10) where there is a gross disparity in acid strength between the two components of the anhydride, but I would expect that, say, P_1-benzyl P^2-di-(*p*-nitrophenyl) pyrophosphate (10; R = benzyl; R′ = *p*-nitrophenyl) could act as a phosphorylating agent, giving with an alcohol a benzyl alkyl phosphate despite the negative charge associated with P_1.

This expectation is based on the established fact that alcohols can be esterified by monoesters of phosphoric acid using roughly stoichiometric quantities by treatment with the chloride of a very strong acid (e.g., an aryl sulphonyl halide) in presence of base.[13] This cannot be due to initial formation of pyrophosphates since diesters of pyrophosphoric acid do not phosphorylate under these conditions. The only satisfactory explanation is that the first step in the reaction is formation of a mixed anhydride (11) which then yields the sulphonate ion and a monomeric metaphosphate; it is this latter which is the true phosphorylating agent. Monomeric

metaphosphates are unknown in the free state since they undergo very rapid polymerisation to polymetaphosphates, but that they would be powerful phosphorylating agents for anions, amines and alcohols can hardly be doubted.



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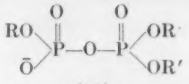

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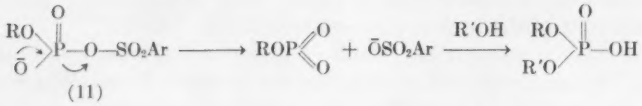

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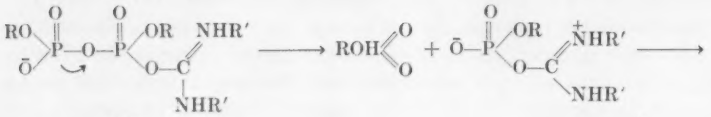

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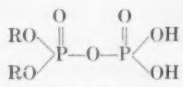


(13)

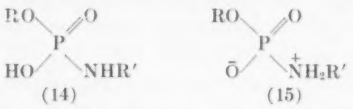
The same explanation can be applied to the observation of Tener *et al.*[14] that polynucleotides may be obtained from mononucleotides by treatment with excess of dicyclohexyl carbodiimide in presence of pyridine. Carbodi-imides are known to convert monoesters of phosphoric acid into P_1P_2-diesters of pyrophosphoric acid (10) rapidly and well-nigh quantitatively.[5] But the pyrophosphate so formed can react with a further molecule of carbodiimide to yield the ψ-urea pyrophosphate which, in its protonated form, is (12). Here is a molecule which will break down to yield 2 mols of monomeric nucleoside metaphosphate from which the production of polynucleotide or phosphorylation of any other alcohol present is readily understandable.

It is suggested that this concept of monomeric metaphosphate as the true phosphorylating agent in phosphorylations based on derivatives of phosphomonoesters provides a coherent picture of the known facts and gets rid of apparent anomalies in them. It is of some interest to note that it also provides an explanation for the fact that a pyridine solution of phosphoryl chloride to which one molar equivalent of water has been added is known to be an effective phosphorylating agent.[15] Addition of water to phosphoryl chloride should produce $HOPOCl_2$ converted by base to the hypothetical chloride of metaphosphoric acid, $ClPO_2$; this one would predict to be a powerful phosphorylating agent rather than a metaphosphorylating agent.

It is also perhaps significant that attempts to prepare P_1-diesters of pyrophosphoric acid (13) by the usual procedures have failed; although doubtless formed they appear to break up at once in water and metaphosphate is in fact always obtained. Production of metaphosphate in this way provides some support for the views outlined above. On these views, too, one might expect that monomeric

metaphosphoric acid would be much less effective than monomeric metaphosphate esters as a phosphorylating agent except in acid solution in its unionised form.

Monoesters of the phosphoramidic acids (e.g., (14)) form an interesting class of phosphorylating agents which belong to this same group.[16, 17] They can, of course, be regarded as "base-metaphosphate complexes" and, in the protonated zwitterionic form (e.g., (15)) are powerful phosphorylating agents for anions; they are indeed among the best reagents for the preparation of pyrophosphates. Their phosphorylating properties would seem most readily explained by breakdown to neutral base and monomeric metaphosphate, although there is admittedly no clear evidence to show that expulsion of the amine precedes rather than accompanies the actual phosphorylation. It should be noted that the value of such a compound as (14) in phosphorylation is affected by the nature of R′ since, in order to be effective, the protonation of the anion must occur on nitrogen rather than on oxygen.



(14) (15)

There are, of course, a few other special phosphorylation reactions which have been described from time to time, but they can be expressed in terms of the various types described above. They include methods depending on the use of imidazole phosphates, of amidine phosphates, of carbonyl *bis*-imidazole as a reagent for preparing pyrophosphates from mono- and di-esters of phosphoric acid, and the use of oxalyl chloride and a phosphomonoester as a reagent for preparing phosphodiesters.

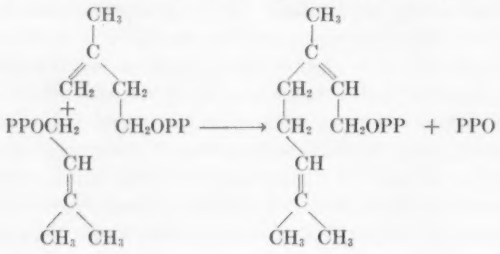
Biological phosphorylation reactions doubtless embrace the same basic types. But whereas, in the laboratory, exchange reactions of polyphosphates are necessarily performed with fully esterified materials, nature commonly operates with mono- or di-esters of polyphosphates (e.g., in the enzymic synthesis of flavin-adenine-dinucleotide) and frequently transfers phosphate rather than esterified phosphate (e.g., in phosphate transfer using adenosine triphosphate). Nature must then have some means of covering up the free acid groups in such compounds producing an effect equivalent to that which can be produced by esterification and so making them highly reactive. To some extent this kind of effect might be produced by complexing with heavy metal ions and, indeed, and activating effect by metal ions on polyphosphates is already known. But a large part may be played by the protein components of the enzyme systems involved in these reactions since strong hydrogen bonding associated with adsorption at the protein surface could produce an effect very similar to that of esterification without any noticeable alteration of pH in the reaction medium as a whole. This idea gains some support from an examination of cyclodextrin inclusion compounds as enzyme models. For example, P_1P_2-diphenyl pyrophosphate is quite stable in aqueous solution buffered at pH 8. but undergoes rapid hydrolysis when β-cyclodextrin (with which it forms an inclusion compound) is added to the solution (F. Cramer and A. R. Todd, unpublished results). The same workers found that adenosine-5′ pyrophosphate similarly forms inclusion compounds with α- and β-cyclodextrins and in this form undergoes ready hydrolysis to adenylic acid and ortho-phosphate. These experiments are only of a preliminary character and require considerable extension, but they offer an approach

to the mechanism of enzyme action which seems to fit many of the known facts and which has the merit of being susceptible to experimental study. The concept has also the advantage of giving some explanation of specificity and of the reason for, say, ADP transferring in some systems phosphate and in other adenylate. The charge distribution in the "activated" pyrophosphate would determine which portion of the molecule would be transferred and also whether the transfer would involve monomeric metaphosphate or not. Some of these problems are now being studied in Cambridge using, *inter alia*, the method of nuclear magnetic resonance spectroscopy.

Formation of Carbon-oxygen, Carbon-nitrogen, Carbon-carbon Bonds.—Although the manifold uses of phosphate and polyphosphate esters in biological processes has long been recognised, attention has, not unnaturally, been focussed mainly on processes in which P-O bonds are made or broken. But they are also concerned in C-O bond formation in the carbohydrate field, in C-N bond formation, and, as the recent beautiful work by Bloch, Lynen and others on biosynthesis of terpenoids, carotenoids and steroids has shown, in C-C bond formation. All these processes are manifestations of the alkylating properties of phosphates to which attention was first clearly drawn by Clark and Todd[18] in connection with studies on the debenzyl-

$$RCH_2O\overset{\overset{O}{\|}}{P}—(OR)_2$$

ation of phosphate esters. Whether a phosphate will alkylate or phosphorylate (i.e., whether a nucleophile will attack the RCH_2 group or the phosphorus) depends primarily on the nature of the esterifying groups. If in such an ester RCH_2 represents a benzyl, allyl or similar group, then treatment with a base or an anion will normally result in alkylation of the base or anion. Again, although tri-*n*-propyl phosphate is not readily dealkylated by bases or salts, mono-dealkylation of *n*-propyl diphenyl phosphate by these means proceeds very easily.[19] An interesting laboratory example of alkylation by phosphates involving carbon-carbon bond formation is provided by the phenol debenzylation procedure for neutral benzyl phosphates in which benzyl groups are removed as *p*-benzylphenol.[20] The role of the phenol here is to act like a reactive olefine. That isopentenyl pyrophosphate should function in biosynthesis as "active isoprene" is thus understandable, provided that the process of synthesis is initiated by one molecule undergoing isomerisation to y:y-dimethyl allyl pyrophosphate. Allowing for the evident capacity of enzyme systems to operate with phosphate and polyphosphate derivatives which are not fully esterified (as has been discussed earlier), y:y-dimethyl allyl pyrophosphate could react with a molecule of *iso*pentenyl pyrophosphate which, as an olefine, is nucleophilic, to yield geranyl pyrophosphate, as indicated in



the scheme above in which PPO represents the pyrophosphate residue. The latter could then either cyclise or react further with a second molecule of *iso*pentenyl pyrophosphate to give farnesyl pyrophosphate, and so on to more complex derivatives. This is, of course, similar to the scheme already postulated by Lynen *et al.*[21] save that actual ionisation to yield a carbonium ion at each stage is not envisaged.

Laboratory evidence which supports this view has been provided by my former colleague, Dr. F. R. Atherton, who has shown that geranyl diphenyl phosphate is spontaneously converted to cyclic terpene and diphenyl phosphate.

This discussion of phosphate chemistry is by no means complete, but enough has been said to indicate in some degree why phosphate derivatives occur so widely as reactive intermediates in biological system. Some of the reactions I have discussed can be simulated by derivatives of other acids, but the tribasic character of phosphoric acid gives it the tremendous flexibility in action which makes it almost ideal as a multi-purpose reagent in living processes.

* Max Tishler Lecture delivered at Harvard University, April 13, 1959.

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*GENETIC RADIATION DAMAGE REVERSAL BY NITROGEN, METHANE, AND ARGON**

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Recently Ebert, Hornsey, and Howard[1, 2] have demonstrated that inert gases can counter and reduce the increase in damage that ordinarily occurs when oxygen is present during irradiation. Gray[3] has discussed his own and other workers' studies of the oxygen effect in radiation damage. The developments following the

reports on inert gases by Ebert *et al.*,[1, 2] and the demonstration by Howard-Flanders[4] that nitric oxide is very similar to oxygen in increasing radiation damage if present during irradiation have increased the complexity of the problems but have opened many new experimental approaches to their solutions. We decided to seek answers to some of these problems by irradiating *Drosophila* in the presence of various combinations of gases in different proportions and observing differences in radiation damage to the different stages of spermatogenesis since some stages are particularly susceptible to radiation damage and to the oxygen effect.

Materials and Methods.—We have tested for sex-linked recessive lethals in *Drosophila melanogaster* and for dominant lethals in *Drosophila virilis*. In these preliminary tests we did not make the more elaborate test for translocations. We used Oregon R for the normal chromosome which was irradiated and the Muller-5 (or *yellow* Muller-5) inversion stocks as the balancer in the test for sex-linked lethals in *melanogaster*. At the time of irradiation the age of the males varied from between 13.5 to 28 hours from eclosion. The males were pretreated in the gas or gas mixtures for 15 minutes (at 1 or 10 atmospheres depending on the test). They were then given 1,000 r irradiation during six minutes with a Westinghouse quadrocondex constant potential machine operated at 250 kv and 15 ma with 0.5-mm Cu and 1-mm Al filters and were post-treated 15 minutes in the gas before the pressure was slowly lowered and the flies etherized and mated to Muller-5. Temperatures during irradiation were 20–21°C. The dosage was determined with a Victoreen r-meter. Repeated runs made each test at the same machine setting and the same distance from the target gave very similar readings. The F_1 females were individually mated to their brothers and the F_2 cultures were scored for lethals. If two or more wild-type males appeared in the F_2, the culture was scored as having a non-lethal irradiated X chromosome; only those cultures with one or no normal males were scored as lethals. The tests were rechecked in F_3.

In the dominant lethal test, *Drosophila virilis* males of strain 1,801.1 from Texmelucan, Mexico, were irradiated 1000 r in 6.0 minutes (tests 1–5) or in 2.4 minutes (tests 6–9), with the same machine settings and pre- and post-treatments of 15 minutes as described for *melanogaster*. The treated males were then mated to heterozygous *virilis* females derived from a cross between strain 2,207.1 from Brazil and strain 2,375.8 from Chile. This pattern of matings insured maximum heterosis, the control tests giving about 96 per cent (92–99 per cent) egg development. Alexander (unpublished data) has shown that the eggs in the control tests which fail to develop are probably not fertilized, since in spot checks for sperm in freshly laid eggs about 4 to 8 per cent fail to show fertilization. The spermatocyte stage (stage G) in *virilis* is exceptional in that sperm may be few following irradiation. No special test was made for stage G, so no correction for lack of fertilization has been made in the calculations. *Drosophila virilis* is used in these tests as there is better synchronization and separation of stages during spermatogenesis than in *melanogaster*.

In order to obtain progeny from sperm that were in the consecutive stages of spermatogenesis during irradiation, the *melanogaster* males were mated to three virgin Muller-5 females after irradiation. At two-day intervals thereafter, the males were placed with a new set of 3 virgin females. Progeny were obtained from each set of females for four days. The males were transferred to new fe-

males six times, giving seven mating periods, A through G. Judging as well as we can from Auerbach's[5] tests in *melanogaster*, stage G represents spermatogonia, F—spermatocytes, E—meiosis, D and C—spermatids, B—sperm bundles and A—sperm. These cell stages may be regarded as synchronized for although there may be some differences in stage in the two day periods most of the cells would be in the stages indicated. However, the actual cytological division stages in meiosis are short so that two-day intervals do not separate all stages in spermatogesis as perfectly as we could desire.

Drosophila virilis develops more slowly than *melanogaster* (Clayton[6]). Males at about 24 hours after eclosion are not sexually mature; thus, after irradiation, these males were placed with 3 females for 5 days to mature. These females were tested but were almost never inseminated. Mating period A comprises the sixth and seventh days, B, the eighth and ninth days, etc., for the ten 2-day mating periods, A through J. We estimate that the progeny from mating periods H, I, and J are the products of spermatogonia at the time of irradiation, G—spermatocytes, F—meiosis, E, D and C—spermatids, B—sperm bundles (and perhaps very late spermatids), and A—sperm bundles (and perhaps not yet motile sperm). In these tests the three females from period A were separated and eggs were counted for four or

TABLE 1

THE FREQUENCY OF SEX-LINKED RECESSIVE LETHALS IN THE PROGENY OF *Drosophila melanogaster* MALES X-RAYED 1000 r IN THE VARIOUS GAS COMBINATIONS AND PRESSURES INDICATED

Gas Mixture	Cultures and Per Cent Lethals	A	B	C	D	E	F	G	Total
10 atm O_2	Nonlethal	231	235	243	180	69	50	181	1,189
	Lethal	10	15	23	24	7	2	5	86
	Lethal, %	4.2	6.0	8.7	11.8	9.2	3.9	2.7	6.7
8 atm N_2 + 2 atm O_2	Nonlethal	212	255	207	168	117	236	234	1,429
(10 atm air)	Lethal	9	9	14	32	13	6	1	84
	Lethal, %	4.1	3.4	6.3	16.0	10.0	2.5	0.4	5.6
8 atm A + 2 atm O_2	Nonlethal	290	295	269	189	110	11	210	1,374
	Lethal	8	8	19	25	10	0	4	74
	Lethal, %	2.7	2.6	6.6	11.7	8.3	0	1.9	5.1
9 atm N_2 + 1 atm O_2	Nonlethal	295	300	296	305	293	293	175	1,957
	Lethal	3	1	4	6	6	6	0	26
	Lethal, %	1.0	0.3	1.3	1.9	2.0	2.0	0	1.3
9 atm A + 1 atm O_2	Nonlethal	212	150	163	199	139	175	162	1,200
	Lethal	2	1	4	8	5	3	3	26
	Lethal, %	0.9	0.7	2.4	3.9	3.5	1.7	1.8	2.1
9 atm CH_4 + 1 atm O_2	Nonlethal	250	245	242	254	206	203	229	1,629
	Lethal	3	3	7	8	6	0	1	28
	Lethal, %	1.2	1.2	2.8	3.1	2.8	0	0.4	1.7
1 atm air (standard condition)	Nonlethal	233	271	249	239	202	90	144	1,428
	Lethal	4	2	13	8	9	1	1	38
	Lethal, %	1.7	0.7	5.0	3.2	4.3	1.1	0.7	2.6
9 atm CO + 1 atm air	Nonlethal	269	261	262	190	123	272	218	1,598
	Lethal	4	10	20	14	7	6	7	68
	Lethal, %	1.5	3.7	7.1	6.9	5.4	2.2	3.1	4.1
9 atm A + 1 atm air	Nonlethal	244	240	247	234	274	239	204	1,682
	Lethal	3	4	3	8	5	3	1	27
	Lethal, %	1.2	1.6	1.2	3.3	1.8	1.2	0.5	1.6
10 atm N_2	Nonlethal	250	293	256	278	238	221	216	1,752
	Lethal	6	6	5	10	6	5	1	39
	Lethal, %	2.3	2.0	1.9	3.5	2.5	2.2	0.5	2.2
10 atm A	Nonlethal	159	148	199	168	158	81	137	1,050
	Lethal	2	1	5	3	3	1	5	20
	Lethal, %	1.2	0.7	2.5	1.8	1.9	1.2	3.5	1.9

five days from one or more of them. The same procedure was followed for females of mating period B, etc. The eggs laid were counted as were the number of pupae that developed from each set of eggs. In *virilis* most lethals act in the egg or larval stages so only a few dominant lethals are missed by scoring pupae. The cycle of events and the variations under different conditions and at different stages have been demonstrated and discussed by Alexander and Stone[7] and by Alexander.[8, 9] Irradiation was performed in 1 atmosphere of air (standard condition) or in 10 atmospheres of gases, singly and in various combinations. The gas environments for the test series are shown in Tables 1 and 2.

TABLE 2

THE EFFECT OF DIFFERENT GASES OR GAS MIXTURES ON THE PRODUCTION OF DOMINANT LETHALS IN *Drosophila virilis* by 1000 r X-RADIATION

Gas Mixtures	Development	A	B	C	D	E	F	G	H	I	J	Total
10 atm O_2, 1,000 r	Eggs	1,540	246	742	851	896	517	468	598	763	658	7,279
	Pupae	786	59	74	75	53	39	196	449	642	622	2,995
	Develop, %	51.0	24.0	10.0	8.8	5.9	7.5	41.9	75.1	84.1	94.5	41.1
10 atm A, 1,000 r	Eggs	1,196	1,730	631	83	153	394	164	...	378	51	4,780
	Pupae	997	1,351	543	54	93	287	108	..	339	37	3,809
	Develop, %	83.4	78.1	86.1	65.1	60.8	72.8	65.9	..	89.7	74.5	79.7
10 atm N_2, 1,000 r	Eggs	742	1,255	233	1,448	677	1,366	911	846	689	466	8,633
	Pupae	614	975	191	850	446	1,058	786	726	578	460	6,684
	Develop, %	82.7	77.7	82.0	58.7	65.9	77.5	86.3	85.8	83.9	98.7	77.4
10 atm CH_4, 1,000 r	Eggs	761	333		152	53*	134*	137*	56*	180	38*	1,844
	Pupae	599	241		97	39	117	126	46	137	29	1,431
	Develop, %	78.7	72.4		63.8	73.6	87.3	92.0	82.1	76.1	76.3	77.6
10 atm CO, 1,000 r	Eggs	655	634	163	830	269	386	14*	69*	67*	192	3,279
	Pupae	487	439	92	424	133	181	8	51	62	146	2,023
	Develop, %	74.4	69.2	56.4	51.1	49.2	46.9	57.1	73.9	92.5	76.0	61.7
2 atm O_2 + 8 atm CH_4, 1,000 r	Eggs	177	144		74	209	123	34	..	..	..	761
	Pupae	130	42		11	41	40	31	..	..	..	295
	Develop, %	73.4	29.2		14.9	19.6	32.5	91.2	..	..	..	38.8
1 atm O_2, 1,000 r	Eggs	426	58	24	373	344	18*	89	212	455	..	1,999
	Pupae	101	14	1	0	0	0	41	101	272	..	530
	Develop, %	23.7	24.1	4.2	0	0	0	46.1	47.6	59.8	..	26.5
1 atm O_2 + 9 atm A 1,000 r	Eggs	189	253	468	162	236	242	55	357	..	222	2,184
	Pupae	159	197	291	52	181	148	45	324	..	198	1,595
	Develop, %	84.1	77.9	62.2	32.1	76.7	61.2	81.8	90.8	..	89.2	73.0
1 atm air + 9 atm CH_4, 1,000 r	Eggs	218	65	42	43	49	223		125	245	..	1,010
	Pupae	144	38	31	22	26	164		96	211	..	732
	Develop, %	66.1	58.5	73.8	52.1	53.1	73.5		76.8	86.1	..	72.5

* Numbers insufficient.

Results—The data showing the sex-linked lethals produced by irradiation in 1 atmosphere of air (standard condition) or in 10 atmospheres of gases or their mixtures during irradiation are given in Table 1 and shown in Figure 1. The data are grouped in terms of the amount of oxygen in the mixture to show interactions between the gases in enhancing or reducing the oxygen effect. Table 1 gives the per cent lethals from each mating period and the total per cent lethals for the test. The total is roughly equivalent to the result that might be obtained from a test where no attempt is made to separate ages and stages.

The data on sex-linked lethals in *melanogaster* show the following relations: 10 atmospheres of oxygen causes somewhat more damage than 8 atmospheres of nitrogen plus 2 oxygen, or 8 argon plus 2 oxygen; all of these cause decidedly more damage by a factor of two or four times than any of the other nitrogen-oxygen, methane-

oxygen, or argon-oxygen mixtures with a smaller amount of oxygen. In fact, the plots for these latter combinations at 10 atmospheres all cluster around those for the pure gases, nitrogen or argon, as far as measurable effect of oxygen on radiation damage is concerned. Moreover, 1 atmosphere of air causes more damage, for example, than 9 atmospheres of nitrogen (or argon or methane) plus 1 atmosphere of oxygen (or air). In contrast to the effect of argon, nitrogen or methane in reducing the damage due to oxygen in suitable mixtures, carbon monoxide increases the amount of damage since 9 atmospheres of carbon monoxide plus 1 of air causes more damage than 1 atmosphere of air when present during irradiation.

The results of the study of the effects of pure gases at 10 atmospheres pressure during irradiation of *virilis* are quite clear (Table 2 and Figure 2). In nitrogen,

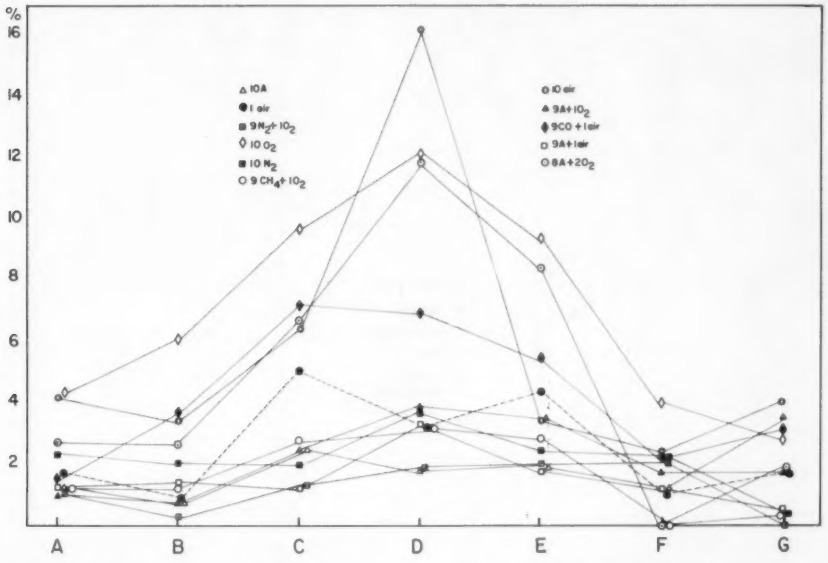

FIG. 1.—The frequency distribution of recessive sex-linked lethals in *Drosophila melanogaster* plotted for the different stages of spermatogenesis for each of the gases. See text for a discussion of stages A–G and other information.

argon, or methane there is between two and three times as much damage in the early spermatids (D and E) as in the sperm bundles and spermatogonia. There are no significant differences between these three gases with the numbers tested; their sum for each stage is approximately equal to that of nitrogen which was the large test. Carbon monoxide (99.5% pure, Matheson) produced additional damage. In fact, the stages C, D, E, F, and G suffered greater damage than any stage with nitrogen, argon, or methane (e.g., E stage in nitrogen). The differences ranged from 10 to 30 per cent in survival. The effect of the presence of 10 atmospheres of oxygen during irradiation is much more drastic. Sperm bundles (or not yet motile sperm), stage A, suffer as much damage as the most susceptible stages in carbon monoxide and suffer greater damage than any stage in nitrogen,

argon, or methane. In fact, we have to go to spermatocytes in G before we reach a stage where equivalent damage occurred even in carbon monoxide and back to spermatogonia, I and J, before the measured damage is equivalent to that in argon, nitrogen, or methane.

The tests with 1 atmosphere of oxygen and with various gas mixtures are given in Table 2 and Figure 3. The damage from 1 atmosphere of oxygen is about the same as from 10 atmospheres. The survival was better at each stage—sometimes markedly so—in 2 atmospheres of oxygen plus 8 atmospheres of methane. With 1

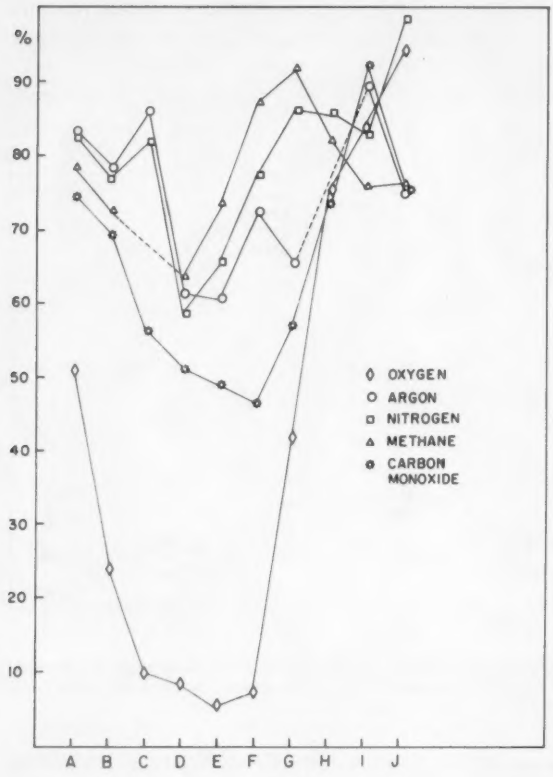

FIG. 2.—The frequency of dominant lethals in *Drosophila virilis* plotted as survival curves. The stages of spermatogenesis and gases are indicated; see text for other information.

atmosphere of oxygen and 9 atmospheres of argon, the damage due to the oxygen is much reduced in all stages—in fact only stage D shows markedly more damage than would occur in 10 atmospheres of argon—but even so the survival in D was much better than in oxygen alone. The test with 1 atmosphere of air plus 9 of methane was small. There seems to be somewhat more damage than in methane alone but no stage was injured as much as D in 1 oxygen plus 9 argon.

Discussion.—This series of preliminary tests provides evidence that the oxygen effect in enhancing radiation damage leading to dominant lethals or sex-linked recessive lethal mutations in *Drosophila* can be countered by nitrogen, argon, or

methane under certain conditions. Presumably most of the dominant lethals are the result of chromosomal abnormalities. We have not determined the proportion of recessive lethals that are associated with visible cytological abnormalities in the *melanogaster* material. Carbon monoxide increases damage above that in nitrogen, argon, or methane or their mixtures with oxygen or air. This may be in part a question of stopping the normal metabolism of oxygen so that internal tissues have the same level of oxygen as those close to the surface (trachea), but part of the

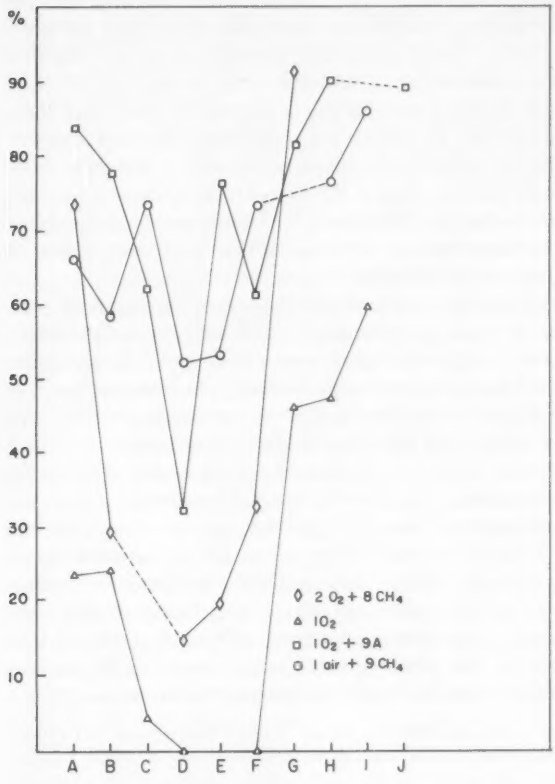

FIG. 3.—The frequency of dominant lethals in *Drosophila virilis* plotted as survival curves. The stages of spermatogenesis and gases are indicated; see text for other information.

damage may result from some other reaction due to carbon monoxide in irradiated cells.

Kihlman[10] showed that if respiration of the broad bean, *Vicia faba*, was blocked during irradiation by cupferron at pH 5.8, maximum chromosome damage (isolocus breaks and exchanges) resulted at 36 μ moles/liter of oxygen. If respiration was not blocked during irradiation, no increase in damage due to the presence of oxygen could be detected until about 65 μ moles/liter were present, and maximum damage required perhaps ten times as much oxygen. Kihlman found an increase in the effect of oxygen and cupferron over that of oxygen without cupferron. We found

an effect of carbon monoxide which seems too high for any residual or metabolic oxygen to account for.

We did not run a series of pressures as did Ebert, Hornsey, and Howard[1, 2], so we could not differentiate between the effectiveness at different pressures of the several gases, argon, nitrogen, or methane, in countering the oxygen effect. Ebert *et al.*[1] showed that cyclopropane counters the oxygen effect and we added methane which seems roughly equivalent to nitrogen or argon in our tests. Ebert and co-workers suggest that the protection may be provided by the displacement of oxygen from some important lipid phase by the protective gas. Their Table 1 showing that the gases with higher lipid/water partition coefficients were more effective at the same gas pressures supports this view. The situation with carbon monoxide is complex since 9 atmospheres of carbon monoxide plus 1 atmosphere of air causes more damage than 1 atmosphere of air (Table 1 and Figure 1) despite the fact that there should be some oxygen displacement by carbon monoxide from the lipid phase at this pressure ratio. Perhaps the simplest tentative conclusion is that very little oxygen is necessary for a large oxygen effect if the cytochrome system is blocked. This is in agreement with the conclusion of Kihlman.[10] There remains the problem of the roles of the oxidative systems through the cytochromes, in addition to that of using up oxygen limited in amount by diffusion.

Dr. A. C. Fabergé proposed that we test methane because of its high lipid solubility. He suggests that the methane or argon might, in dissolving and partitioning between chromosomes and the surrounding aqueous phase, make chromosomes less accessible to the water and thus protect them to some extent from free radicals or other active radiation products in the surrounding or permeating water. Alternatively the production of active lipid peroxides might be diminished.

Summary.—The oxygen effect, leading to increased radiation damage, can be countered or prevented to a large degree by the addition of 9 atmospheres of argon, nitrogen, or methane to 1 atmosphere of air or oxygen during irradiation. On the other hand, the addition of 9 atmospheres of carbon monoxide to 1 atmosphere of air increases the radiation damage. These findings apply to dominant lethals produced in *Drosophila virilis* and sex-linked recessive lethals produced in *Drosophila melanogaster*. The stages of spermatogenesis from spermatogonia to mature sperm differ among themselves in their response to the same amount of X-radiation and to the oxygen effect and its reduction or enhancement by the other gases.

* This work was supported by a contract with the Atomic Energy Commission [AT-(40-1)-1323], by a grant from the Rockefeller Foundation, and a grant from the Robert A. Welch Foundation.

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*STUDIES ON NORMAL AND GENETICALLY ALTERED TRYPTOPHAN SYNTHETASE FROM NEUROSPORA CRASSA**

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Recent experiments have implicated indole-3-glycerol phosphate (InGP) as an intermediate in the conversion of anthranilic acid to tryptophan in *Escherichia coli*[1] and *Neurospora crassa*.[2, 3] In addition, Yanofsky[4] has demonstrated that a single enzyme, trytophan synthetase, from *E. coli* catalyzes the following three reactions:

$$\text{indole} + \text{serine} \rightarrow \text{tryptophan} \quad (1)$$

$$\text{InGP} + \text{serine} \rightarrow \text{tryptophan} + \text{triose phosphate} \quad (2)$$

$$\text{InGP} \rightleftharpoons \text{indole} + \text{triose phosphate} \quad (3)$$

Reaction (1) has been studied in some detail in extracts from *Neurospora crassa*.[5] This reaction requires the presence of pyridoxal phosphate. Further studies demonstrated that extracts from wild type *N. crassa* also catalyze reactions (2) and (3), and suggested that InGP is converted to tryptophan by wild type *N. crassa* without the formation of free indole.[2, 3]

While a majority of *N. crassa* auxotrophs which lack tryptophan synthetase (td mutants) accumulate indole glycerol in their growth filtrate, two accumulate indole.[6] If the final step in tryptophan biosynthesis is the conversion of InGP to tryptophan, the accumulation of indole by these td mutants cannot be explained by the absence of a single enzyme.

The following data indicate that the accumulation of indole by td mutants is attributable to the presence of an altered tryptophan synthetase, which catalyzes reaction (3) but not reactions (1) or (2).

Materials and Methods.—Mutant td 2, which was previously studied,[7] and td 71, which was obtained by X-irradiation of the wild type strain 74A,[6] were used. Both of these mutants require tryptophan for growth and accumulate indole in their culture filtrates, while all other td mutants which have been isolated accumulate indole glycerol in their growth medium. Both td 2 and td 71 have been shown to be allelic with other td mutants.[6]

Wild type 74A, which has been used for these studies, was grown on minimal medium[8] and mutant strains td 2 and td 71 were grown on minimal medium plus 100 μg L-tryptophan per ml. Cultures were grown on a rotary shaker at 30°C for 48–72 hours. Mycelia were filtered through cheesecloth, washed and lyophilized. The lyophilized mycelia were powdered in a Wiley mill and stored at −15°C.

Extracts were prepared at 4°C by grinding one part powdered, lyophilized mycelia with two parts alumina A-301 with the gradual addition of 15 parts of 0.05 *M* phosphate buffer, pH 7.8. The extract was centrifuged at 100,000 × *g* for 30 min and the supernatant used as the crude extract. The crude extract always contained 15–20 mg of protein per ml.

Fractionation procedures were carried out for either concentration or purification of enzyme activity and were the same for both wild type and mutant strains. To

concentrate the activity in crude extracts, 14 ml of 1.5 per cent protamine sulfate was added to 100 ml of extract. The mixture was stirred for 10 min and then centrifuged at 20,000 × *g*. Solid ammonium sulfate was added to the supernatant to a concentration of 65 per cent saturation. The precipitate formed was collected by centrifugation at 20,000 × *g* for 15 min and dissolved in 10–20 ml of 0.05 *M* phosphate buffer, pH 7.8. This preparation was used as the concentrated extract.

Purification of activity was carried out as follows: After protamine sulfate treatment, the supernatant was brought to 50 per cent saturation with solid ammonium sulfate, mixed for 10 min and centrifuged at 20,000 × *g* for 15 min. The precipitate was dissolved in 10.0 ml of 0.025 *M* phosphate buffer, pH 7.8 (Step I). This preparation was diluted with 20.0 ml of 0.05 *M* phosphate buffer, pH 7.0, and mixed with 9.0 ml of alumina C γ gel, pH 7.0, for 10 min. The mixture was centrifuged at 20,000 × *g* for 10 min and the precipitate discarded (Step II). The supernatant was then fractionated with a saturated solution of ammonium sulfate. The precipitate which formed between 25 and 30 per cent saturation with ammonium sulfate was collected by centrifugation and dissolved in 3.0 ml of 0.05 *M* phosphate buffer, pH 7.8 (Step III). This fraction was designated fractionated extract and usually exhibited a 45–50 fold increase in specific activity over the crude extract with about 25–30 per cent recovery. All the fractionation operations were carried out at 0–5°C.

Reaction (1) was measured by the disappearance of indole.[9] Reaction (2) was determined by the appearance of tryptophan by a modification of a procedure by Kupfer.[10] Assays for reaction (1) and (2) in mutant extracts were set up to detect at least 1 per cent of the wild type activity level. Reaction (3) was assayed by indole formation using the same procedure employed in the measurement of reaction (1). The reverse reaction, InGP formation from indole, was measured by the periodate method of Yanofsky.[1] CRM (cross reacting material) was determined according to the procedure of Suskind.[11] Protein was assayed with the Folin reagent.[12]

Results.—Activities of wild type tryptophan synthetase:—Early studies provided relatively convincing evidence that indole was an intermediate product in tryptophan biosynthesis and was the substrate for tryptophan synthetase.[13, 9] However, the demonstration that extracts from *Neurospora* are capable of catalyzing reactions (1), (2), and (3), and the evidence for the participation of a single enzyme in these three reactions in *E. coli* casts some doubt on the previous interpretations. An attempt was made, therefore, to establish whether these reactions were catalyzed by one, or more than one enzyme in *Neurospora*. Table 1 shows the data obtained when the three activities (reaction (1), (2), and (3)) were followed over a 34-fold purification range of the wild type enzyme. The ratio of the three activities remains essentially constant within the limits of the assay procedures employed. Further, it was found that the three activities are eluted from a DEAE cellulose ion exchange column in the same fractions. These results indicate that a single protein, tryptophan synthetase, is responsible for reactions (1), (2), and (3).

It may be concluded that tryptophan synthetase is responsible for the formation of indole as well as tryptophan. The absence of tryptophan synthetase from td 2 and td 71 extracts, as judged by the absence of reaction (1), and the accumulation of indole by these mutants would therefore appear to be contradictory.

TABLE 1

FRACTIONATION OF WILD TYPE ACTIVITIES*

Fraction	Reaction (2) μM/hr/mg pr	Ratio reaction (1)/-reaction (2)	Ratio reaction (3)/-reaction (2)
Crude extract	0.055	1.8	0.016
Step I*	0.172	1.5	0.014
Step II	0.209	1.7	...
Step III	1.89	1.3	0.020

* See *Methods* for fractions steps.
† For assay of reaction (2), the complete system contained 25 μM phosphate buffer, pH 7.8, 20 μg B_6P, 0.1 μM InGP, 80 μM DL-serine and, 0.05 μM glutathione in a final column of 1.0 ml. The complete system for the assay of reaction (3) is the same as in Table 4. The complete system for the assay of reaction (1) is that of Yanofsky.[9]

Activities of indole accumulating mutants:—Crude extracts from td 2 and td 71 as well as 74A were examined for their ability to convert InGP to indole. When the extracts were properly supplemented (see below), the reaction was found in td 2 and td 71 as well as wild type, but not in td 6 which is an indole glycerol accumulating td mutant (Table 2). Reaction (3) could be detected in td 2 extracts only when the

TABLE 2

RELATIVE LEVEL OF REACTION (3) IN VARIOUS CRUDE EXTRACTS UNDER OPTIMAL CONDITIONS

Extract	μM/hr/mg pr*
74A (Wild type)	0.0011
td 2	0.0030
td 71	0.040
td 6	0.0000

* The assay system for each strain is the same as the complete systems shown in Table 3. The td 6 extract was assayed with the complete system used for td 71 extract.

extracts were concentrated by ammonium sulfate precipitation so that large quantities of protein could be used. This procedure also had to be used routinely for the detection of reaction (3) activity in wild type extracts. The specific activities obtained with td 2 extracts were of the same order of magnitude as the reaction in crude extracts of wild type. In td 71, on the other hand, a relatively high level of activity was obtained. Reaction (1) and (2) could not be demonstrated in crude or purified preparations of extract from td 2 and td 71. It appeared, therefore, that these mutants had lost the ability to catalyze two of the reactions catalyzed by wild type tryptophan synthetase, but still retained the third, i.e., InGP → indole.

Data demonstrating the requirements for the conversion of InGP to indole by extracts from the three strains in Table 3. Surprisingly, in each case a different set of requirements was found. In the case of wild type extract, the reaction was always measured in the absence of serine to avoid the complication of simultaneous tryptophan formation. It can be seen, however, that pyridoxal phosphate is not

TABLE 3

REQUIREMENTS FOR CONVERSION OF INGP TO INDOLE

	μM/hr/mg protein		
	74A*	td 2†	td 71‡
Complete§	0.017	0.003	0.040
minus InGP	0.000	0.000	0.001
minus B_6P	0.032	0.001	0.007
minus Serine	...	0.003	0.007

* 74A—fractionated extract (see *Methods*).
† Td 2—concentrated extract (see *Methods*).
‡ Td 71—crude extract.
§ The complete system for 74A contained 25 μM phosphate buffer, pH 7.8, 20 μg B_6P and 0.2 μM InGP in a final volume of 1.0 ml. For td 2 and td 71, 80 μM DL-serine was added. The incubation was for 1 hr at 37°C.

required by the wild type and is, in fact, somewhat inhibitory. In td 2 extracts, the reaction requires pyridoxal phosphate for maximum indole formation, but serine does not stimulate the reaction. The reaction in td 71 extracts requires both serine and pyridoxal phosphate for the maximum rate in the crude extracts and an absolute requirement for each could be demonstrated in more purified extracts.

The reverse reaction, indole + triose phosphate → InGP, could be demonstrated with each preparation if, in addition to indole, hexose diphosphate and aldolase were added as a source of triosephosphate. For each strain the requirements were the same as for the conversion of InGP to indole.

Relationship of activities to tryptophan synthetase: Many of the td mutants (including td 2 and td 71) produce a protein which is immunologically related to tryptophan synthetase of the parental wild type.[14, 6] This protein appears to be mutationally altered tryptophan synthetase and has been designated CRM (cross reacting material).

When td 2 extract is chromatographed on a DEAE cellulose ion exchange column, enzyme activity is eluted in the same fraction as the main CRM peak. In addition, Suskind and Jordan[15] have demonstrated that the enzymatic conversion of indole to InGP by td 2 extracts is inhibited by anti-CRM serum which has been absorbed by an extract of a non-CRM forming mutant.

The activity from td 71 has not been successfully chromatographed on DEAE cellulose columns, but after a 35-fold purification of the enzyme activity by conventional fractionation procedures, the ratio of CRM to enzyme activity remains constant (Table 4).

TABLE 4

RATIO OF CRM TO ENZYME ACTIVITY IN td 71 EXTRACTS

	μM/hr/mg pr	Units CRM*/mg pr	Ratio CRM/enz. act.
Crude extract	0.044	0.172	3.9
Fractionated extract	1.57	5.45	3.5

* 1 unit of CRM is defined as that amount of material which will neutralize the antibody which would inhibit 1 μM/hr of reaction (1) of wild type enzyme.

The data lend strong support to the suggestion that, in mutants td 2 and td 71, reaction (3) is catalyzed by a protein which is identical to CRM.

Characteristics of td 71 activity: Mutant td 71 has chosen for further study since it is isogenic with the wild type strain 74A. In addition, the higher activity of reaction (3) in td 71 extracts makes this mutant much more suitable for enzyme studies.

The reaction characteristics in td 71 are of interest, since all the substrates required for the conversion of InGP to tryptophan or of indole to tryptophan with wild type enzyme, are required for the conversion of InGP to indole by this mutant. This is true in spite of the fact that neither serine nor pyridoxal phosphate plays an obvious role in the enzymatic conversion in question. To investigate the possible nature of the functional alteration which has taken place, the affinity constants for serine and pyridoxal phosphate were compared to those for the wild type enzyme. The fractionated extract was used in each case. For this purpose wild type activity was assayed by reaction (1) and td 71 activity was assayed by reaction (3). The affinity constants for serine (K_A for td 71 and K_m for wild type)

were calculated from the data in Figure 1. The K_A for serine for reaction (3) in td 71 appears to be essentially the same as the K_m for serine for reaction (1) in 74A. On the other hand, the saturation curves for pyridoxal phosphate for the respective reactions are quite different (Fig. 2). The concentration of pyridoxal phosphate required for half saturation is at least 10-fold higher in the td 71 reaction. It appears that the mutational alteration which results in a loss of reactions (1) and (2) and which creates new requirements for reaction (3) also alters the pyridoxal phosphate combining site in some way. This effect on the affinity of the enzyme

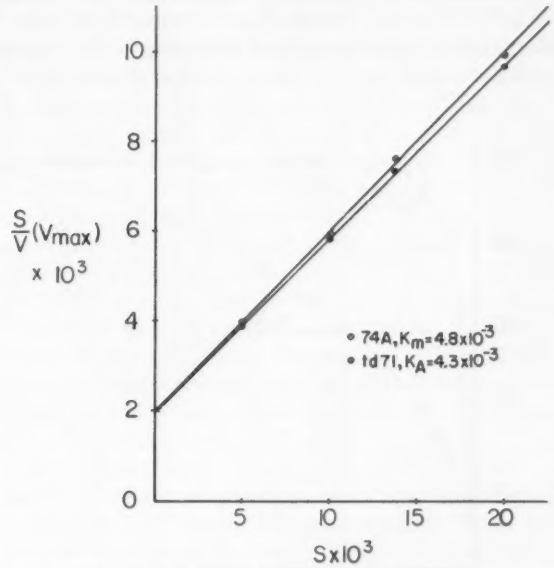

FIG. 1.—The affinity of enzyme from 74A and td 71 for serine. 74A was assayed by reaction (1) according to the procedure of Yanofsky.[9] Reaction (3) was assayed in td 71 using the complete system in Table 3. S represents the concentration of L-serine in 1.0 ml. and V the velocity of the reaction in μM per hr. Each point represents the average of determinations carried out for 3 different time intervals. The ordinate is plotted as S (V max)/V so that both curves have the same intercept and any difference in the affinity constants should be reflected in the slopes.

for pyridoxal phosphate is apparently not paralleled by an effect on the affinity of the enzyme for serine.

Discussion.—The chromatography of a *Neurospora* tryptophan synthetase on DEAE cellulose columns[16] indicates that this enzyme is a one-component system as opposed to the two-component protein system demonstrated with tryptophan synthetase from *E. coli* by Crawford and Yanofsky.[17] The single component nature of the enzyme is even more dramatically demonstrated by the relatively high state of purity obtained recently with this enzyme by Mohler and Suskind.[18] The fractionation studies presented here indicate that tryptophan synthetase in *N. crassa*, as in *E. coli*, catalyzes reactions (1), (2), and (3).

Td mutants which retain the ability to catalyze one of the tryptophan synthetase

reactions, either (1) or (3), have been isolated from *E. coli* and characterized by Yanofsky and Crawford.[19] The mutants studied here grossly resemble one of these classes. However, the requirement for pyridoxal phosphate or serine for the catalysis of reaction (3) is not exhibited by any of the *E. coli* mutants. The alterations found in td 2 and td 71 present two cases of mutation which have resulted in retention of part of the catalytic function of an enzyme, and at the same time imposed new requirements for the part of the mechanism retained.

These requirements in td 2 and td 71 pose some interesting questions regarding enzymatic mechanism since serine and pyridoxal phosphate play no obvious role in the conversion of InGP to indole. Application of current hypotheses,[20] concerning the role of pyridoxal phosphate in amino acid reactions, to the conversion of InGP or indole to tryptophan would invoke the formation of a Schiff's base between

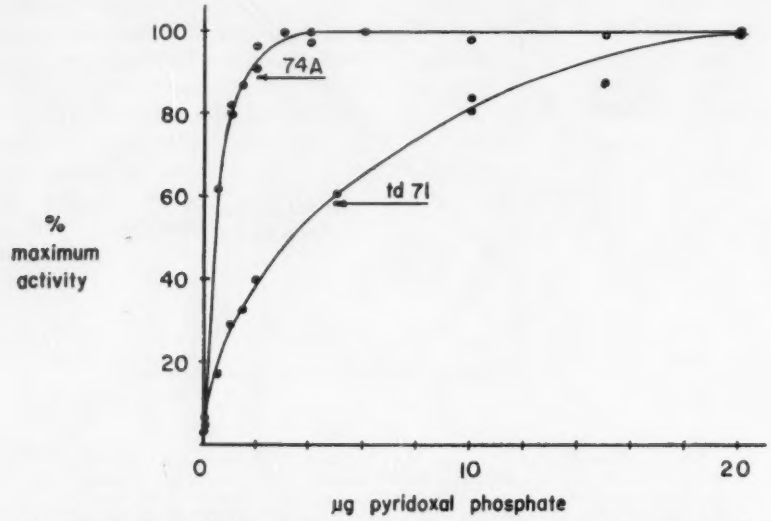

FIG. 2.—The effect of B_6P concentration on 74A and td 71 reactions. 74A was assayed by reaction (1) and td 71 reaction (3).

pyridoxal phosphate and serine on the enzyme surface which would react with the indole moiety to form tryptophan. According to this concept, the Schiff's base would not be directly involved in the conversion of InGP to indole. The absence of any requirement for pyridoxal phosphate for this conversion in wild type and the requirement for pyridoxal phosphate alone in td 2 would appear to justify this conclusion. It is possible that serine or pyridoxal phosphate are required for reaction (3) in td 2 and td 71 to maintain the active site of the altered enzyme in its "proper" physicochemical or stereospecific configuration to combine with and cleave InGP. The "induced fit" theory for enzyme action of Koshland[21] can easily be extended to explain such an activation.

It is interesting to consider what structural change could lead to the functional alterations observed. The td 71 enzyme apparently retains the ability to combine

with all of the substrates of the wild type enzyme but still fails to carry out the over-all reaction. Therefore, a simple loss or block of one of the substrate combining sites cannot be postulated. It appears that the structural alteration blocks the ability of the indole moiety to react with serine without blocking the combining sites or without blocking the catalysis of reaction (3). More information is required concerning the mechanism of the wild type enzyme conversion before any specific conclusions can be drawn. It is already apparent, however, that a detailed study of the structural and functional alterations involved in the td 2 and td 71 enzymes should provide significant information on the effect of mutation on enzyme function.

The alterations in the td 2 and td 71 enzymes are clearly different, although the gross change in the enzyme reaction is similar. The relative levels of reaction (3) present in the three strains constitute another obvious difference. The level of activity in td 2 extracts is low and is approximately that found in the wild type extracts. In addition, the CRM to enzyme activity ratio is similar in td 2 and wild type (approximately 100/1, where the units of reaction (1) are assumed to be equivalent to the units of CRM). However, in td 71 extracts the specific activity is high and approximates very closely the specific activity of reaction (2), InGP → tryptophan, found in similar preparations of wild type. The CRM to enzyme activity ratio in td 71 extracts is only about 4 to 1. Thus, not only have the requirements for this reaction been changed as a result of the mutation but there is an apparent increase in the rate of the reaction per unit of CRM activity. Alternately, a change in immunological reactivity of the protein may be involved.

Rachmeler and Yanofsky[22] have recently obtained a td mutant of *Neurospora* which is capable of catalyzing reaction (1), indole → tryptophan, but not reactions (2) or (3). A detailed analysis of the alteration involved in such a mutant should provide an interesting comparative study to the data presented here.

Summary.—Only two of the many td mutants isolated from *Neurospora crassa* accumulate indole in their growth filtrates. While wild type tryptophan synthetase catalyzes reactions (1), (2), and (3), extracts from these two mutants, td 2 and td 71, catalyze reaction (3), but not reactions (1) or (2). Experimental evidence to date indicates that reaction (3), in extracts from td 2 and td 71, is catalyzed by a protein (CRM) which is immunologically related to wild type tryptophan synthetase. It is therefore suggested that the reaction is catalyzed by a mutationally altered tryptophan synthetase in these mutants.

These studies indicate that, while the ability to carry out reaction (3) is retained, something more subtle than loss of combining sites for serine and for pyridoxal phosphate is involved in the loss of reactions (1) and (2). While reaction (3) in wild type does not require pyridoxal phosphate and serine for the conversion of InGP to indole, reaction (3) in td 2 requires pyridoxal phosphate and reaction (3) in td 71 requires both pyridoxal phosphate and serine. Thus, in addition to loss of ability to catalyze reactions (1) and (2), the altered enzymes have acquired some new requirements for the reaction retained.

In more detailed studies with td 71 extracts, it was found that the association constant for serine is very similar to the K_m for serine in the wild type reaction. On the other hand, the concentration curves for pyridoxal phosphate are significantly different for the td 71 and the wild type reactions.

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*MEIOTIC DRIVE IN NATURAL POPULATIONS OF DROSOPHILA MELANOGASTER. II. GENETIC VARIATION AT THE SEGREGATION-DISTORTER LOCUS**

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In a previous report, Sandler, Hiraizumi, and Sandler[1] have presented an account of the elementary observations concerning the phenomenon of segregation-distortion. Briefly, these observations are as follows: *Segregation-distorter* (symbol *SD*) is a locus, located in or near the centromeric heterochromatin of chromosome II, which was discovered in a natural population of *D. melanogaster*. When *SD* is made heterozygous with a structurally normal, non-*SD*-bearing, second chromosome in a male, the *SD*-bearing chromosome is recovered very much more often than its (SD^+-bearing) homolog. If the ratio of *SD*-carrying progeny to the total progeny in a backcross, $SD/+$ ♂♂ × $+/+$ ♀♀, is called k, then, in many experiments, the average k value is 0.95 or greater. When, however, *SD* is made heterozygous in males carrying, as a homolog for *SD*, a structurally rearranged SD^+-bearing chromosome (as, for example *In* (*2LR*) *Cy*); the phenomenon is suppressed, and the k value is approximately 0.50. Data of this kind have been interpreted to mean that an *SD* locus must pair with an SD^+ locus in order for segregation-distortion to occur. In SD/SD^+ females, it should be mentioned, there is no segregation-distortion under any circumstances.

The evidence (genetic and cytological) thus far obtained is consistent with the following hypothesis about the cytogenetic basis of segregation-distortion.[1] When *SD* synapses with SD^+, in a male, the formal equivalent of a chromosome fracture and reversed sister-chromatid reunion occurs somewhere in the SD^+-bearing chromosome (perhaps at the SD^+ locus). This process somehow causes the cells involved in the resultant anaphase II bridge-like structure to proceed abnormally through spermiogenesis with the consequence that functional SD^+-bearing sperm are not produced. It is this meiotically conditioned failure to transmit the SD^+ chromosome that accounts for the distorted segregation ratios from heterozygous *SD* males.

It has now been found that the proportion of heterozygous *SD* males resulting from any given cross which exhibits segregation-distortion, and the amount of distortion that any particular male shows (the *k* value), varies widely depending upon the precise source and history of the *SD*-bearing and the SD^+-bearing chromosomes in the heterozygotes being tested. It is the purpose of this report to present a general account of the kinds of variations in the behavior of *SD* which have been observed. More fully documented reports of each phase of the work are currently in preparation, and will be presented elsewhere. For this reason, extended discussion of these variations and comparable systems in other organisms[2-4] has been omitted.

Origin of SD Lines.—The original *SD* lines collected in nature were of two types. One of these, typified by a line designated *SD-5*,[5] contains (1) two inversions in IIR which almost completely eliminate crossing over in that arm and (2) a lethal that is so far inseparable from the more proximal of the two inversions. The other type is represented by one line—*SD-72*—which contains only the more distal inversion in IIR, is lethal free, and shows a slightly higher (though still much lower than normal) rate of crossing over in IIR. From crosses of females carrying some one of these *SD* chromosomes and a normal chromosome marked by the mutants *cn* and *bw* (*cinnabar* near the base and *brown* near the tip of IIR), occasional recombinants between *cn* and *bw* are recovered. Recombinants from *SD-72* are symbolized *R(SD)-n*[6] and fall into only two classes: (1) phenotypically *cn*, non-*SD*, carrying the *SD-72* inversion, and (2) phenotypically *bw*, *SD*, carrying no gross inversions. From one *SD-5* type line (*SD-36*) one *bw* recombinant (symbolized here *R(SD-36)-1*) carrying *SD*, the lethal, and both inversions has been obtained. In one rather small experiment (with a total of only 1,246 F_1), eight recombinants (5 *cn* and 4 *bw*) were recovered from *SD-5*. This high rate of crossing over is not understood and has never repeated itself in either the *SD-5* line itself or in the inversion-containing recombinants themselves. These recombinants are symbolized *R(SD-5)-n*.[7] The *cn* recombinants in this case are non-*SD*, and carry neither inversion nor the lethal; the *bw* recombinants are *SD* and carry the lethal and both inversions.

The behavior of *SD* in crosses depends, in part, upon which particular *SD* line is used. It may be noted that all of these lines have been repeatedly backcrossed to *cn bw* females for at least ten generations (and in most cases for many more) and therefore, except for chromosome II, have the genetic background of the *cn bw* strain.

Unstable SD Lines.—When any of the six originally isolated *SD* lines is tested for distortion against a laboratory *cn bw* stock (which has been used throughout as a

"standard tester" stock), in crosses of single *SD/cn bw* males by *cn bw* females, the *k* value for each such male tested is very close to the average *k* value which itself is almost invariably higher than 0.90. Figure 1 is a typical histogram of the *k* values from experiments using lines of the *SD-5* type. When, however, *SD*-bearing recombinants from any source are tested, the situation is very different in that only about one-half of the recombinants (referred to as "stable") show distributions of *k* values similar to those of the original lines. The other half (called "unstable") show an extremely wide variation in the *k* values of individual males. A typical example of an unstable recombinant is *R(SD-36)-1*. Crosses of males heterozygous for this recombinant and the standard *cn bw* chromosome by *cn bw* females yield a distribution of *k* values illustrated in Figure 2A. This figure may be compared with Figure 1. Sons of *R(SD-36)-1* males showing *k* values higher than 0.90 and lower than 0.75 were also tested. The results are shown diagrammatically in

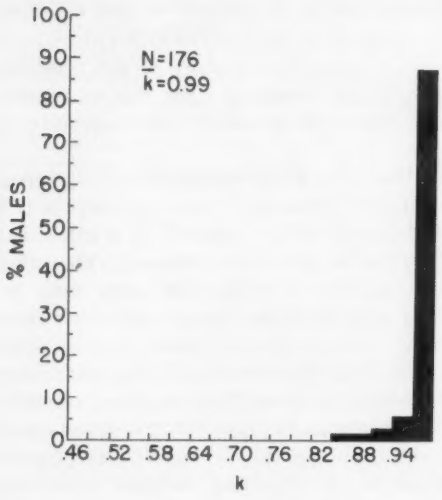

FIG. 1.—The frequency distribution of *k* values in a series of crosses of *SD/cn bw* ♂♂ × *cn bw/cn bw* ♀♀. The *SD* chromosomes are of the *SD-5* type. *N* = total number of males tested; $\bar{k}$ = unweighted mean *k*. The number of progeny per tested male was about 100.

Figures 2B and 2C respectively. It can be seen that, except for a slight displacement of the mean *k* value, there is little effect of the parental *k* value on the *k* values of the male offspring. In general, selection for either high or low *k* values is weakly effective (in the sense of changing the unweighted mean *k* value) for one generation of selection, but ineffective thereafter. Indeed, unstable *SD* lines have been selected, during routine stock transfers, for high *k* values for at least 25 generations with no appreciable change in the frequency distribution of *k* values. Formally it is as though unstable *SD* alleles can exist in two states, one of which is characterized by a high *k* value, the other by a low *k* value, but that the "gonial mutation rate" (meaning here merely the probability of a chromosome passing through meiosis carrying *SD* in the alternative state to the state the male in question received) is high—but less than one-half. This means that the distribution of *k* values from a family of sib males depends to some extent on the *k* value of the father but for a particular father would be independent of the *k* value of the grandfather.

The above discussion considered only the question of what instability means—not its genetic basis. The latter is a peculiarly troublesome matter. At first sight, it would appear that, since about one half of the recombinants between *cn* and *bw* are unstable, there is a stabilizer locus elsewhere on the right arm of chromosome II which is necessary for stability. There are, however, two immediate lines of evidence indicating that the situation is more complex than this. First, the stable *SD*-bearing recombinants from *SD-72* have lost the inversion and presumably all of the material distal to it, which means that the stabilizer must be between *SD* and the proximal breakpoint of the *SD-72* inversion. The stable *SD*-bearing recombinants from *SD-5* and *SD-36*, on the other hand, carry this inversion (in addition to the proximal one) so that the exchanges in these cases would appear to have taken place between the distal breakpoint of the distal inversion and the locus of *bw*, which would put the stabilizer in that region. From this it follows that if

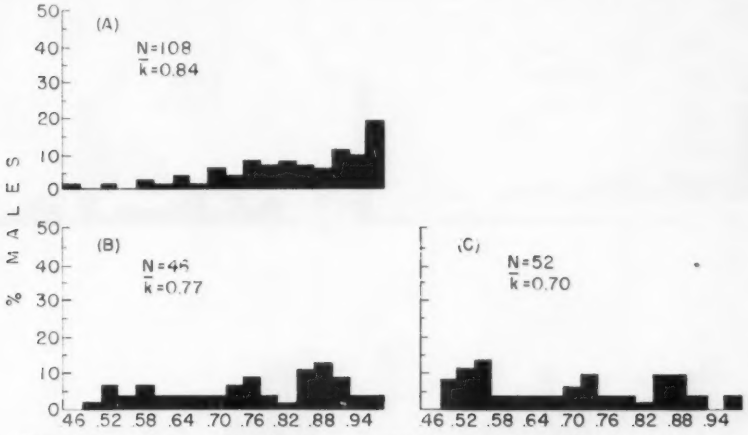

FIG. 2.—The frequency distribution of k values in crosses of *R(SD-36)-1/cn bw* ♂♂ × *cn bw/cn bw* ♀♀. (*A*) Parental k values unknown; (*B*) Parental $k > 0.90$; (*C*) Parental $k < 0.75$.

stabilizer locus is to account for instability, then either (1) there are at least two positions for such loci—one on *SD-72* and another on the *SD-5* type lines—or (2) the origin of these recombinants, though apparently simple, is really more complex than at present imagined. A second set of observations difficult to reconcile with a simple stabilizer locus is this: even the so-called "stable" recombinants are slightly less stable than the original lines from which they were derived. It may even be (although it is very difficult to demonstrate this) that different unstable recombinants are different, one from the other.

As a consequence of these observations, it appears an unavoidable conclusion that either (*a*) there is a complex of interacting stabilizer loci, or perhaps two stabilizer loci, each capable of existing in a number of states—which is *a priori* improbable—or (*b*) that instability represents a change at the *SD* locus itself. In this case, however, it is difficult to understand why unstable lines are always recombinants. This question has yet to be resolved.

Insensitive SD^+ Alleles and the Phenomenon of Translocal Modification.—The type of result diagrammed in Figure 1 is typical of the original *SD* lines and similar to results obtained from the stable recombinant *SD* lines when either is tested heterozygous with the standard *cn bw* tester chromosome. It is found, however, that the distribution of *k* values is different for different SD^+ chromosomes—there being, however, a distinct and repeatable distribution for each SD/SD^+ combination. We may speak of those SD^+ chromosomes associated with reduced *k* values as being relatively *insensitive* to the distorting action of *SD*. In this sense, the standard *cn bw* chromosome is the most sensitive thus far tested. Many intermediate levels of sensitivity have been found, ranging from one *cn bw* stock (not the standard, of course) which shows about ten per cent somewhat reduced *k* values with *SD-5*, to a bw^D stock which is completely insensitive to *SD-5*. Histograms of the frequency distribution of *k* values from a series of representative SD^+ lines are shown in Figure 3.

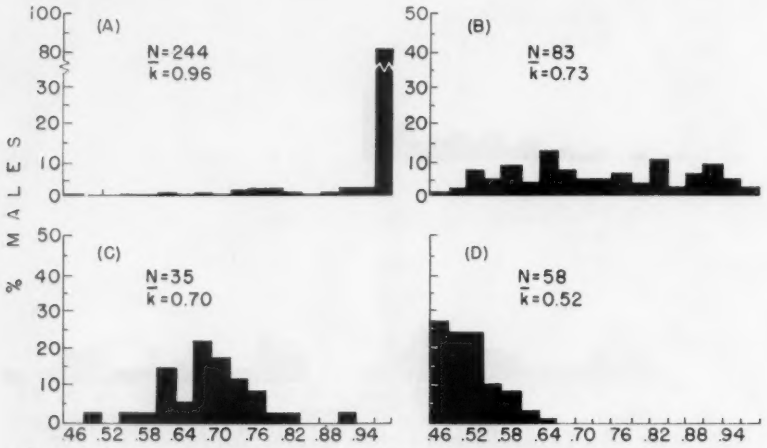

FIG. 3.—The frequency distribution of *k* values in crosses of males carrying *SD-5* heterozygous with SD^+-bearing homologs of differing levels of sensitivity. The stocks used are: (*A*) *cn bw; e;* (*B*) *al; ru;* (*C*) *bw; e; ey²;* (*D*) bw^D; *st*.

The first question of concern in this connection is whether this insensitivity is a consequence of modifier loci or a consequence of the SD^+ alleles themselves. It seems almost certain that the latter is correct.

It has, first of all, been shown for one line of intermediate sensitivity (represented in Figure 3C) that the level of sensitivity segregates with chromosome II, so that independent modifier loci for this case can be ruled out.

Secondly, an experiment was performed in which the standard *cn bw* chromosome was made heterozygous with the completely insensitive bw^D chromosome in females, and *cn* and cn^+ chromosomes were recovered. These chromosomes were then tested for sensitivity to *SD-5* and it was found that the *cn* chromosomes were almost always sensitive while the cn^+ chromosomes were almost always relatively insensitive. It is known[1] that the *SD* locus is very close to the locus of *cn*, so that the *cn* chromosomes in this experiment derived their SD^+ alleles predominantly from the

sensitive *cn bw* chromosome while the cn^+ chromosomes contain SD^+ alleles predominantly from the insensitive bw^D chromosome.

The demonstration that insensitivity is a property of the SD^+ allele has, however, led to the discovery of a new phenomenon in this system. If a homozygous bw^D fly (of either sex) is mated to a homozygous standard *cn bw* fly and resultant *cn bw*/bw^D heterozygous males crossed to *SD-5* females, two classes of *SD* heterozygous males are obtained: *SD-5*/*cn bw* and *SD-5*/bw^D. When these are tested, it is found that the sensitivity of a substantial proportion of the SD^+ alleles on the standard *cn bw* chromosome have now become less sensitive. A typical experiment of this sort is diagrammed in Figure 4. If, furthermore, *SD-5*/*cn bw* sons of those *SD-5*/*cn bw* and *SD-5*/bw^D males showing a low k value in this experiment are themselves tested, it is found that there is a partial—but not complete—return to the normal k value. This result is also shown in Figure 4. This change in the *SD-5*/*cn bw* males appar-

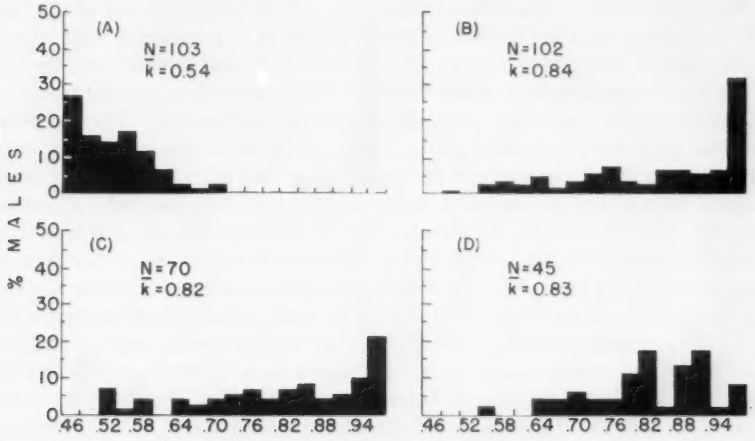

FIG. 4.—The frequency distribution of k values from crosses of: (*A*) *cn bw*/bw^D ♂♂ × *SD-5* ♀♀ → *SD-5*/bw^D ♂♂ × *cn bw* ♀♀; (*B*) *cn bw*/bw^D ♂♂ × *SD-5* ♀♀ → *SD-5*/*cn bw* ♂♂ × *cn bw* ♀♀; (*C*) *SD-5*/*cn bw* ♂♂, showing reduced k values in (*A*), by *cn bw* ♀♀; (*D*) *SD-5*/*cn bw* ♂♂, showing reduced k values in (*B*), by *cn bw* ♀♀.

ently means that the changed SD^+ in *cn bw* has in turn changed the *SD* allele of *SD-5*. The *SD-5* chromosomes recovered from *SD-5*/bw^D males are also changed in their effect on the unchanged *cn bw* SD^+ allele (Figure 4C). These, directed, partial, changes of the state of SD^+ and *SD* alleles will be referred to as "translocal modifications."

Numerous other examples of the phenomenon of translocal modification have been found. Not only does the completely insensitive SD^+ allele of the bw^D chromosome translocally modify, but SD^+ alleles of intermediate sensitivity also have this effect. It is interesting that SD^+ alleles of intermediate sensitivity also translocally modify to a lesser degree than the completely insensitive SD^+ allele.

Figure 4 showed that the insensitive SD^+ allele was apparently not affected by one generation passage heterozygous with the sensitive SD^+ allele. This insensitive allele can, however, be made more sensitive by translocal modification (even to

giving occasional k values in excess of 0.90) by repeated passage with the standard SD^+; that is, by crossing *cn bw/bw*^{p} heterozygotes to the standard *cn bw* line repeatedly and testing the *bw*^{p} chromosome for sensitivity to *SD-5*. Here it can be shown that as the insensitive SD^+ becomes more and more sensitive (both with respect to the k value exhibited by any given male and with respect to the proportion of k values significantly higher than 0.50), its power to translocally modify the standard SD^+ is reduced. It appears, in general, that the degree of translocal modification depends upon the degree of sensitivity or insensitivity of the modifying SD^+ allele when tested for its ability to modify an allele of some constant sensitivity.

It can, moreover, be shown that the insensitive SD^+ allele of *bw*^{p} is translocally modified in a single passage heterozygous with *cn bw* by crossing *bw*^{p}*/cn bw* males to *SD-5;* XXY females and testing the *SD-5/bw*^{p} sons. This is almost certainly related to the fact that *SD* alleles from XXY females are "stronger" distorters than *SD* alleles from XX females. (See section on "Conditional Distortion.")

The Phenomenon of Conditional Distortion.—With respect to any of the original *SD* lines or any of the *SD*-bearing recombinants, it is possible to make recurrent backcrosses of the type *SD/cn bw* ♂♂ × *cn bw* ♀♀, and to determine the k value of the *SD/cn bw* males in each generation. When this is done, the distribution of k values is, in every case, very much like that shown in Figure 1. The exceptions to this rule have been mentioned above: these are (1) if the recombinant is unstable and (2) even if the recombinant is "stable" it is still slightly less stable than the original lines so that occasional k values of 0.75–0.85 are observed. Considering, however, only the original *SD* lines and the stable recombinants, it is true that the k values observed remain uniformly high throughout a pedigree of the type under consideration. If, however, we consider a pedigree of another type, the situation is very different. In particular, if an *SD/cn bw* male (whose *SD* chromosome can be any stable recombinant from *SD-72;* that is, lacking lethals and inversions and marked by the mutant allele of *bw*) is crossed to a female carrying *In(2LR)Cy* (which, it should be mentioned, behaves with respect to translocal modification exactly as does the standard *cn bw* chromosome) and the F_1 *SD/Cy* females are collected and crossed to *cn bw* males so that, in the F_2, *SD/cn bw* males are obtained, there are produced two types of sibships. One type is composed only of males showing the typical uniformly high k values. The other type of sibship contains two classes of males with equal frequency: (1) males showing the usual, high, k value, and (2) males showing no distortion (i.e., showing a k value of approximately 0.50). A female producing a male sibship characterized by only high k values is referred to as unconditionally distorting, or *unconditioned;* a female producing both distorting and nondistorting males is called *conditioned.* A typical experiment illustrating the phenomenon of "conditional distortion" is shown in Figure 5. Also shown in that figure is the fact that the sons of males which showed no distortion do themselves distort normally. That is, passage of a stable *SD*-bearing recombinant (from *SD-72*) through a conditioned female for a single generation produces, in one half the cases, *SD/cn bw* males which do not distort. Passage of these same chromosomes, however, through a male for one additional generation completely restores their ability to distort. The behavior of these *SD* lines after two or more generations passage in females has yet to be adequately studied.

Although conditional distortion is obviously easiest to distinguish unambiguously

in stable *SD* lines, it has, in fact, been found in all seven *SD*-bearing recombinants from *SD-72*. These all seem to be, at least qualitatively, the same in this respect. In the case of *SD-72*, itself, the phenomenon of conditional distortion appears to occur but it manifests itself somewhat differently. Here, it appears as if that half of the sons of a conditioned female which would have shown no distortion in the case of the recombinants, show a reduced distortion in the case of *SD-72;* that is, about one half of the sons of a conditioned female show the usual high k value, the other half show k values of about 0.70 to 0.80. In the case of the *SD-5* type original lines, this phenomenon is either absent or the low k values are sufficiently high as to be individually indistinguishable from the high k values. Some pedigrees of *SD-5* suggest that the latter may well be the case. The only recombinant from *SD-5* type lines thus far examined for conditional distortion is the unstable *R(SD-36)-1* which behaves exactly like *SD-5* (or *SD-36*) in this respect.

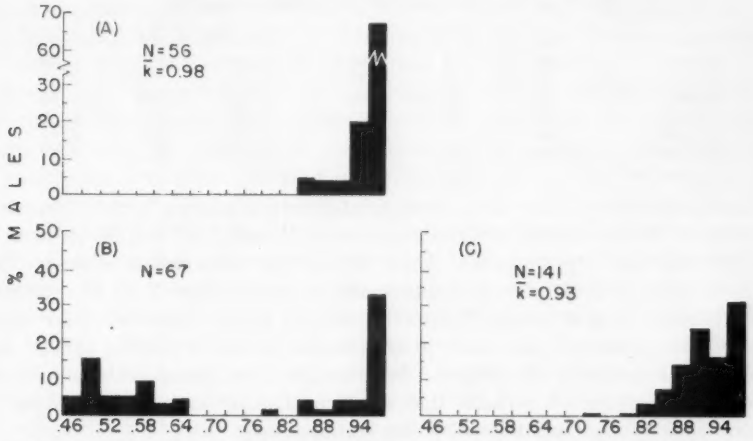

FIG. 5.—The frequency distribution of k values from crosses of: (*A*) *R(SD)-4/cn bw* ♂♂ from *R(SD)-4/In(2LR)Cy* ♂♂ × *cn bw* ♀♀; (*B*) *R(SD)-4/cn bw* ♂♂ from *R(SD)-4/In(2LR)Cy* ♀♀ × *cn bw* ♂♂; (*C*) *R(SD)-4/cn bw* ♂♂ from *R(SD)-4/cn bw* ♂♂ × *cn bw* ♀♀, which showed no distortion in (*B*).

In summary then, all *SD*-bearing chromosomes lacking inversions exhibit conditional distortion; *SD-72* which has only the distal inversion apparently produces conditioned females, but the sons of these females show an intermediate level of distortion suppression, while the sons of conditioned females carrying those *SD* chromosomes with the two inversions show either no, or a very low, level of distortion suppression. The precise relationship of the inversions to conditional distortion has yet to be examined experimentally.

It is worth noting at this point that two anomalies in the original report on *segregation-distortion*[1] are now resolved. In the first place, two experiments were there reported in which *SD* was passed only through males in one case (Table 1 of that work) and through females, testing sons in each generation, in the other case (Table 2 of that work). The average k values from those experiments are given in Table 1 here. The difference in k values noted are undoubtedly due to conditional

distortion. This is evidence, also, for the point made above: namely, that even in the *SD-5* type lines this phenomenon occurs although the manifestation is much less extreme. A second anomaly now resolved concerns the *k* values of the recombinants recovered as females from *SD-72/cn bw* females. In these cases, certain of the recombinants showed low *k* values. This was initially attributed to crossing over between the *SD* locus and cn^+ in the recovered recombinant female. A direct mapping, on the other hand, indicated that *SD* and cn^+ are almost inseparable (see also below). It is now clear that the low *k* values observed from a mixture of distortable and nondistortable sons from the recombinant females, due to the passage of *SD* through a female.

TABLE 1

THE AVERAGE *k* VALUES FROM (A) FOUR GENERATIONS OF RECURRENT BACK CROSSES OF SD/*cn bw* ♂♂ × *cn bw* ♀♀, AND (B) THREE GENERATIONS OF RECURRENT BACK CROSSES OF *SD/cn bw* ♀♀ × *cn bw* ♂♂ (TESTING THE *SD/cn bw* MALE PROGENY IN EACH SUCH GENERATION)

	A			B		
Line	SD	SD^+	k	SD	SD^+	k
SD-1	1,551	43	0.97	1,979	117	0.94
SD-5	1,506	118	0.93	1,771	170	0.91
SD-8	1,779	22	0.99	1,761	282	0.86
SD-9	1,376	87	0.94	1,794	298	0.86
SD-36	1,304	37	0.97	1,902	255	0.88
SD-72	1,700	48	0.97	1,352	401	0.77

Another observation relevant to conditional distortion which is both of theoretical interest and of considerable technical importance is that XXY females produce no nondistorting sons, irrespective of which *SD*-bearing chromosome is used. This has been tested thus far only in females carrying an attached-X (of two different types, however) and a normal Y chromosome. It is not completely clear at the present time, therefore, just what, in an attached-X and Y-bearing female, suppresses the phenomenon of conditional distortion, but it is clear that the suppression occurs. Furthermore, it appears that conditional distortion can be modified in various ways by manipulations of the sex chromosomes.

Finally, it is interesting to note that the fraction of *SD* chromosomes, from a female, that do not distort the standard *cn bw* (SD^+) chromosome, are nevertheless not themselves distorted by a distorting *SD* chromosome. This is easily tested by crossing a female carrying a stable *SD*-bearing recombinant to males of the constitution *SD-5/cn bw*, and collecting males of the constitutions: (1) *R(SD)/cn bw* and (2) *R(SD)/SD-5*. In those sibships in which one-half of the *R(SD)/cn bw* males did not distort, the ratio of *R(SD)* to *SD-5* progeny from their *R(SD)/SD-5* brothers was still 1:1.

On the Relations Among the Different SD States.—There have been presented four ways in which the characteristic *k* value of certain *SD*-bearing chromosomes may be reduced. These are: (1) if the *SD* allele is unstable; (2) if the *SD* allele is tested against an insensitive SD^+ allele; (3) by translocal modification; and (4) by passage through a conditioned female. It seems apparent that these four mechanisms, as well as the resultant *SD* states, though undoubtedly related, are all distinct. That the mechanisms are distinct is, of course, direct. Instability is a permanent property of an *SD* line, insensitivity refers to the SD^+ allele, translocal modification is a temporary state imposed on the *SD* allele by the SD^+ allele, and

conditional distortion depends upon being passed through a conditioned female lacking a Y chromosome. Instability, it might be mentioned, is independent of the sex of the parent contributing the *SD* allele to the tester male, and also independent of whether or not an extra Y chromosome is present. The phenomena, other than instability, are themselves pairwise distinct by the criteria of (*a*) whether *SD* or SD^+ is affected, (*b*) whether the *k* values are independent of the sex of the parent contributing the *SD*-bearing chromosome or not, and (*c*) the frequency distribution of *k* values characteristic of each phenomenon.

If, moreover, attention is fixed upon some particular *k* value when measured against the standard SD^+ (in particular, a *k* value of approximately 0.50), the question of whether these are distinct states with the same characteristic *k* value or the same state produced by different mechanisms can be answered by considering the subsequent behavior of the nondistorting *SD* states. In the case of instability, there is only a return to the characteristic unstable pattern. In the case of translocal modification there is a slow return to the characteristic distribution of *k* values, generation by generation. In the case of conditional distortion, there is immediate recovery after one additional generation passage through a male.

It may, finally, be worth noting that translocal modification of one SD^+ allele by another of a different sensitivity is an effect involving normal (i.e., non-*SD*-bearing) chromosomes only. In this regard, the phenomenon of segregation-distortion is only a detection mechanism for what must be a general kind of directed genetic change.

On the Physical Nature of the SD Locus.—The physical basis of the phenomenon of segregation-distortion is a genetic locus which maps close to the centromere of chromosome II. Crossover tests on *SD*-bearing recombinants from *SD-72* (i.e., recombinants containing no gross inversions by salivary gland analysis) give roughly normal map lengths for the entire second chromosome. As the number of identifiable *SD* states multiplied, however, it became increasingly interesting to try to find some operational property of the *SD* locus other than distortion itself. For this reason, a crossover test was performed in which crossing over between the closely linked (3 standard crossover units apart) markers, *pr* and *cn*, was measured in (*a*) heterozygous *SD* and (*b*) homozygous SD^+ females. The *SD* locus is between *pr* and *cn* as is the centromere of chromosome II. The results of these tests are given in Table 2. It can be seen that *SD* dramatically reduces crossing over in its immediate vicinity.

This raises the possibility that *SD* is a small structural change in the centromeric heterochromatin, and the different *SD* states are not mutations, in the sense of

TABLE 2

THE RESULTS FROM CROSSES OF FEMALES HETEROZYGOUS FOR *pr* AND *cn* WITH AND WITHOUT *SD*, BY HOMOZYGOUS *pr cn* MALES

Phenotype of Progeny	Constitution of Parental Female—	
	pr cn/+	pr cn/SD
pr cn	1,465	1,581
+	1,530	1,689
pr	17	1*
cn	17	0
% crossovers	1.12	0.03

* This recombinant was SD^+.

informational changes, but are structural changes. It is not difficult, for example, to imagine that the synapsis of *SD* and SD^+ is different for different structural states of *SD* (and perhaps SD^+) which differences are reflected as changes in the distribution of k values.

Summary.—When a chromosome II carrying an *SD* allele is made heterozygous with certain structurally normal SD^+-bearing second chromosomes in a *D. melanogaster* male, the *SD*-bearing chromosome is recovered much more frequently than its SD^+-bearing homolog. The extent of the excess recovery of *SD* (the k value), and the proportion of males showing any specific amount of excess, depends on the source and history of the *SD* and SD^+-bearing chromosomes. In particular: (1) there are "unstable" *SD* lines which regularly produce males characterized by k values anywhere between 0.50 and 1.00, generation after generation; (2) there are SD^+ alleles of various "sensitivities" to any given *SD* ranging from a completely insensitive allele (producing a k value of 0.50 against any *SD*) to the very sensitive "standard" SD^+ allele with an average k value well above 0.90 in standard backcrosses; (3) the sensitivity of an SD^+ allele (as well as the ability to distort of an *SD* allele) can be changed ("translocal modification") by making the allele in question heterozygous with an insensitive allele for one or more generations (and conversely); and, finally, (4) when certain *SD*-bearing chromosomes are passed for one generation through a female, so balanced that crossing over in the vicinity of *SD* does not occur, a proportion of such females produce two types of heterozygous *SD* sons about equally frequently: (*a*) those showing normal distortion and (*b*) those showing no distortion. Females of this type are referred to as "conditioned" and the phenomenon as "conditional distortion."

Finally, it is observed that crossing over in the immediate vicinity of the *SD* locus is very much reduced suggesting, perhaps, that *SD* is a localized structural modification of some sort, and the various *SD* states departures from some standard structural condition.

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[5] The *SD-5* type lines are: *SD-1*, *−5*, *−8*, *−9*, and *−36*.

[6] Recombinants *R(SD)-2*, *−4*, *−5*, *−6*, *−7*, *−8*, and *−9* are *bw*; *R(SD)-3*, *−10*, *−11*, and *−12* are *cn*.

[7] Recombinants *R(SD-5)-17*, *−18*, *−21*, and *−22* are *bw*; *R(SD-5)-15*, *−16*, *−19*, and *−20* are *cn*. One *cn* recombinant was lost before tests could be made.

*ON THE SPECTRAL THEORY OF STRONGLY ELLIPTIC DIFFERENTIAL OPERATORS**

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In a recent note in these Proceedings,[1] we have discussed existence theorems for a general class of boundary-value problems for elliptic partial differential equations, basing our argument upon general *a priori* estimates for the L^p and Hölder norms for the highest order derivatives appearing in the differential operator. We mentioned at the beginning of that discussion that the techniques which we were applying yielded much sharper information for the case of the Dirichlet problem for a strongly elliptic operator (and indeed, for a whole class of related problems which we shall describe in another place). It is the purpose of the present note to develop some of these sharper results. As before, we make minimal assumptions on the continuity and regularity of the coefficients of our differential operator, and obtain results both in the case of bounded and unbounded domains.[2]

Let $A = \sum_{|\alpha| < 2m} a_\alpha(x) D^\alpha$ be a differential operator of order $2m$, $(m \geq 1)$, whose coefficients are complex-valued functions defined on an open subset G of the Euclidean n-space E^n with boundary Γ. Here, as in our preceding note, for each ordered n-tuple $\alpha = (\alpha_1, \ldots, \alpha_n)$ of nonnegative integers, we set $D^\alpha = [(1/i)\ (\partial/\partial x_n)]^{\alpha_1} \ldots [(1/i)\ (\partial/\partial x_n)]^{\alpha_n}$, $|\alpha| = \sum_{j=1}^{n} \alpha_j$. For each n-vector $\xi = (\xi_1, \ldots, \xi_n)$ we set $\xi^\alpha = ((\xi_1)^{\alpha_1} \ldots, (\xi_n)^{\alpha_n})$. The characteristic form $a(x, \xi)$ of A is defined for x in G and any real n-vector ξ by

$$a(x, \xi) = \sum_{|\alpha| = 2m} a_\alpha(x) \xi^\alpha.$$

The partial differential operator A is said to be uniformly strongly elliptic on G if there exists a constant $c_1 > 0$ such that $Re\{a(x, \xi)\} \geq c_1 |\xi|^{2m}$ for all x in G and all real n-vectors ξ.[3] A will be said to be essentially real if the top-order coefficients a_α for $|\alpha| = 2m$ are real-valued functions on G.

Our basic regularity assumptions on the coefficients of A and on the domain G are the following:

(I) G is uniformly regular of class C^{2m}. (For the details of the definition of uniform regularity of G, we refer to our preceding note.[1])

(II) The coefficients a_α of A are uniformly bounded on G for all α.

(III) The top-order coefficients a_α of A for $|\alpha| = 2m$ are uniformly continuous on G.

The formal adjoint A' of A is defined in general as a differential operator with coefficients which are distributions of order at most $2m - 1$, by

$$A'u = \sum_{|\alpha| \leq 2m} D^\alpha(\bar{a}_\alpha(x) u) = \sum_{|\alpha| \leq 2m} a_\alpha'(x) D^\alpha,$$

for u in $C_c^\infty(G)$, the family of infinitely differentiable functions with compact support in G. The coefficients of A' can be expressed by the Leibniz formula as linear combinations of derivatives of the $\bar{a}_\alpha$. In some, though not all, of our results, we shall utilize the following assumption on the coefficients a_α':

(IV) There exists a constant $M > 0$ such that $|a_\alpha'(x)| \leq M$ for all α, almost everywhere in G.

We are interested in studying the spectrum and the resolvent of the differential operator A realized in $L^2(G)$ (and more generally in $L^p(G)$ for $1 < p < \infty$) with domain consisting of functions satisfying null Dirichlet boundary conditions on Γ in a suitable extended sense.

We recall the definition of the Dirichlet problem in its precise terms. We consider the Sobolev space $W^{2m,\,p}(G)$ which, for each p with $1 < p < \infty$, consists of all u in $L^p(G)$ for which all the distribution derivatives $D^\alpha u$ also lie in $L^p(G)$ for $|\alpha| \leq 2m$. This linear subset of $L^p(G)$ becomes a reflexive Banach space with respect to the norm $\|u\|_{2m,\,p} = \{\sum_{|\alpha| \leq 2m} \|D^\alpha u\|^p_{L^p(G)}\}^{1/p}$. Under the assumption of regularity (I) upon the domain G, it follows that, if $R(G)$ is the subfamily of $W^{2m,\,p}(G)$ consisting of the restrictions to G of infinitely differentiable functions with compact support in E^n, then $R(G)$ is dense in $W^{2m,\,p}(G)$, and in addition, the mapping which assigns to each function u in $R(G)$ the corresponding function $D^\beta u|_\Gamma$ on Γ for a fixed β with $|\beta| < 2m$, is a bounded linear mapping from the dense subset $R(G)$ of $W^{2m,\,p}(G)$ into $L^p(\Gamma)$, where the latter space is taken with respect to the $(n-1)$-dimensional volume induced on Γ by E^n. If we continue to denote the unique continuous extension of this linear map to a linear mapping from $W^{2m,\,p}(G)$ into $L^p(\Gamma)$ by $D^\beta|_\Gamma$, we may formulate the following definition:

Definition 1.—The domain of A_p, $1 < p < \infty$, i.e., the domain of the differential operator A realized in $L^p(G)$ under null Dirichlet boundary conditions, is the family of all u in $W^{2m,\,p}(G)$ for which $D^\beta u|_\Gamma = 0$ for $|\beta| < m$. For such u, we set $A_p u = Au$, the latter obviously being a well-defined element of $L^p(G)$, and A_p is given as a linear transformation from $D(A_p) \subset L^p(G)$ into $L^p(G)$.

Remark 1: The second condition on the vanishing of the extended derivatives of order $< m$ on the boundary of G is equivalent, as follows from an easy argument, to the following condition which is similar to the generalized Dirichlet null-condition considered in the variational treatment of the Dirichlet problem: For each point x in Γ, there is a neighborhood N of x in E^n and a sequence of function $\{\psi_j\}$ from $R(G)$, each vanishing identically on a neighborhood of $N \cap \Gamma$, such that $\|D^\beta(u - \psi_j)\|_{L^p(N)} \to 0$ as $j \to \infty$ for all β with $|\beta| \leq m$.

The basic *a priori* estimate upon which our discussion is based is the following: There exists a constant $k_p > 0$, depending only on the constant of uniform ellipticity c_1, the bounds of (II) and the modulus of continuity in (III) as well as p, such that for u in $D(A_p)$

$$\|u\|_{2m,\,p} \leq k_p\{\|Au\|_{L^p(G)} + \|u\|_{L^p(G)}\}.^4 \tag{1}$$

Remark 2: The inequality (1) implies immediately that A_p is a closed operator from $L^p(G)$ into $L^p(G)$. Indeed, for a single differential operator A (omitting the dependence of k_p upon the regularity conditions), it is equivalent by the closed graph theorem to the fact that A_p is closed.

Our objective in the discussion which follows is to prove the following theorems:

THEOREM 1.—*Let A be an uniformly elliptic partial differential operator of order $2m$ on a domain G of E^n, with A and G satisfying the regularity conditions (I), (II), and (III) above. Then:*

(a) *Given $\epsilon > 0$, there exists $k(\epsilon) > 0$, such that for all real numbers ξ and all u in $D(A_2)$,*

$$\|(A + \xi I)u\|_{L^2(G)} \geq \{(1 - \epsilon)\xi - k(\epsilon)\}\|u\|_{L^2(G)}.$$

(b) *If, in addition, the domain G is bounded, the resolvent set of A_p, for every p with $1 < p < \infty$, eventually contains every ray in the complex plane emanating from the origin and making an angle of less than $\pi/2$ with the negative real axis.*

(c) *If in addition, G being bounded or unbounded, the coefficients of the top order terms a_α of A ($|\alpha| = 2m$) satisfy an uniform Holder condition on G, then there exists a constant λ with $0 < \lambda < 1$ and a constant $k_1 > 0$ such that for all u in $D(A_2)$, ($\xi \geq 0$),*

$$\|(A_2 + \xi I)u\|_{L^2(G)} \geq \{\xi - k_1\xi^\lambda - k_1\}\|u\|_{L^2(G)}.$$

In this case, if G is bounded, then for ζ on the spectrum of A_p,

$$Re(\zeta) \geq -k_2(1 + |Im(\zeta)|^{\lambda'}),$$

where λ' is a constant with $0 < \lambda' < 1$.

(d) *Suppose that the coefficients of the adjoint operator A' to A satisfy the regularity assumption (IV) above, Γ locally of class $4m$, and that our previous hypotheses are verified. Then, G being bounded or unbounded, the resolvent set of A_2 contains a left half-plane $Re(\zeta) \geq k$, for some constant k,* and *on the real axis for $\xi \geq k$,*

$$\|(A_2 - \xi I)^{-1}\| \leq (\xi - k)^{-1}.$$

THEOREM 2. *Suppose that A and G satisfy condition (I), (II), and (III), and that G is bounded. Then for ξ large and negative, $(A_p - \xi I)^{-1}$ exists and is a compact linear transformation in $L^p(G)$. In particular, the spectrum of A_p is discrete, the eigenspaces are of finite dimension, and $(A_p - \zeta I)^{-1}$ exists if $(A_p - \zeta I)$ has a trivial null-space.*

THEOREM 3. *Suppose that A and G satisfy the regularity conditions (I), (II), and (III), where A is uniformly elliptic and essentially real on G. Then given $\epsilon > 0$, there exists $k(\epsilon) > 0$ such that for ζ complex with $Re(\zeta) \geq 0$, $u \in D(A_2)$,*

$$\|(A_2 + \zeta I)u\|_{L^2(G)} \geq \{(1 - \epsilon)|\zeta| - k(\epsilon)\}\|u\|_{L^2(G)}.$$

In particular, if G is bounded, the spectrum of A_p for all p with $1 < p < \infty$, lies in a fixed half-plane $Re(\zeta) \geq k_4$.

THEOREM 4. *Suppose that A is uniformly elliptic and essentially real on a domain G of E^n, with A and G satisfying the regularity conditions (I), (II), (III), and (IV), and Γ locally of class $4m$. Then:*

(a) *There exists a constant $k_{5,p} > 0$ such that for all p with $0 < p < \infty$, $\lambda_1 = [(2m - 1)/2m]$, the spectrum of A_p is contained in the set*

$$\{\zeta: |Im(\zeta)| \leq k_{5,p}(1 + Re(\zeta)^{\lambda_1}), Re(\zeta) \geq -k_5\}.$$

(b) *For all ζ with $Re\ (\zeta) \leq 0$, $|\zeta| > k_6$,*

$$\|(A_2 - \zeta I)^{-1}\| \leq \{|\zeta| - k_6|\zeta|^{\lambda_2} - k_6\}^{-1},$$

where $\lambda_2 = [(2m - 1)/(2m + 1)]$.

We shall prove Theorems 1, 2, and 3 in their general form, but Theorem 4 only for $p = 2$. The proof of the latter result for general p will be given in another

place. For these proofs, we shall make use of Theorems 5 and 6 below, which we have previously announced and whose proofs we sketch below. Some of the details of the proof of Theorems 5(*b*) were given in ref. 1 (essentially the complete proof for $p = 2$, or for G bounded) and we present only that portion of the argument for general p and G which was omitted there for lack of space.

THEOREM 5.—(*a*) *Suppose that A is uniformly elliptic on G with A and G satisfying the regularity conditions (I), (II), and (III). Let N_p be the null space of A_p. Then $N_p < N_{p_1}$ if $p < p_1$ equality holding if G is bounded.*

(*b*) *Suppose that A is uniformly elliptic on G with A and G satisfying the regularity conditions (I), (II), (III), and (IV), Γ locally of class $4m$. Let $1 < p, q, (p^{-1} + q^{-1} = 1)$, and let A_p and A'_q be the realizations of A and A' under null Dirichlet boundary conditions in $L^p(G)$ and $L^q(G)$, respectively. Then $(A_p)^* = A'_q$.*

Proof of Theorem 5(a): This follows from the sharper formulation of the *a priori* estimates as given in our preceding note.[1]

Proof of 5(b): Since under the hypotheses of 5(*b*), A_p and A'_q are closed operators and the operators A and A' are interchangeable, it suffices to consider the case $q \geq 2$. It suffices, moreover, to show that if u lies in the domain of $(A_p)^*$, then u lies locally in $W^{2m,\,q}$ on a neighborhood of each point of $G \cup \Gamma$. We proceed in two stages: (1) We show that u lies locally in $W^{2m,\,2}$; (2) If u lies locally in $W^{2m,\,2}$ and if $A'u$ lies in $L^q(G)$, then if u satisfies the null Dirichlet conditions in the $W^{2m,\,2}$ sense locally at each point of Γ, then u lies locally in $W^{2m,\,q}$.

For the proof of (1), let G_1 be a bounded uniformly regular subdomain of G having a portion Γ' of its boundary Γ_1 contained in Γ. We define two operators $A_{2,\,0}$ and $A'_{2,\,1}$ in $L^2(G_1)$ as follows:

$$D(A_{2,\,0}) = \{u: u \in W^{2m,\,2}(G_1);\ D^\beta u\,|_{\Gamma'} = 0 \text{ for } |\beta| < m;\ D^\beta u\,|_{\Gamma_1 - \Gamma'} = 0 \text{ for } |\beta| < 2m\},$$

$$A_{2,\,0}u = Au, \text{ for } u \text{ in } D(A_{2,\,0});$$

$$D(A'_{2,\,1}) = \{u: u \text{ lies in } W^{2m,\,2} \text{ locally in a neighborhood of every point of } G_1 \cup \Gamma',\ A'u \text{ lies in } L^2(G_1);\ D^\beta u\,|_{\Gamma'} = 0 \text{ for } |\beta| < m\};$$

$$A'_{2,\,1}u = A'u, \text{ for } u \text{ in } D(A'_{2,\,1}).$$

The operators $A_{2,\,0}$ and $A'_{2,\,1}$ are closed operators in $L^2(G_1)$, as follows from simple variants of our *a* priori estimates. Moreover, the restriction of our given function u from $D(A_p^*)$ to G_1 clearly lies in the domain of $A^*_{2,\,0}$. It therefore suffices to show that $A^*_{2,\,0} = A'_{2,\,1}$. By the procedure of our preceding note, we may assume without loss of generality that the coefficients of A and A' are infinitely differentiable functions on G_1. It suffices by the argument of our preceding note to show that $A^*_{2,\,0}A_{2,\,0} = A'_{2,\,1}A_{2,\,0}$ and that $(A'_{2,\,1})^*A'_{2,\,1} = A_{2,\,0}A'_{2,\,1}$.

The argument for the second equality is the simpler, since if v lies in the domain of $(A'_{2,\,1})^*A'_{2,\,1}$, then $A'_{2,\,1}v$ lies in the domain of the operator $(A'_{2,\,1})^*$ and, since the operator A_2' (taken for the domain G_1) is contained in $A'_{2,\,1}$, $(A'_{2,\,1})^* \subset (A_2')^* = A_2$. But if $A'_{2,\,1}v = v_1$ lies in $D(A_2)$ and if in addition $(v_1, A'_{2,\,1}w) = (f, w)$ for some f in $L^2(G_1)$ and all w in $D(A'_{2,\,1})$, the same equality persists if we set $v_1 = 0$ outside G_1 and consider all w in $D(A')$. It follows immediately that v_1 satisfies the null Cauchy boundary conditions on $\Gamma_1 - \Gamma'$, and hence that v lies in $D(A_{2,\,0}A'_{2,\,1})$.

For the first equality, we observe that if v lies in $D(A^*_{2,0}A_{2,0})$, then v lies in $D(A_{2,0})$ and $(A_{2,0}v, A_{2,0}w) = (f, w)$ for some f in $L^2(G_1)$ and all w in $D(A_{2,0})$. In other words, v is a solution of the variational boundary-value problem corresponding to the coercive form $(A_{2,0}v, A_{2,0}v)$ on the subspace $D(A_{2,0})$ on $W^{2m,2}(G_1)$, (the coerciveness being a consequence of the *a priori* estimate in $W^{2m,2}(G_1)$). By the regularity theory for solutions of variational boundary-value problems, v lies in $W^{4m,2}(G_1)$ and therefore $A_{2,0}v$ lies in $D(A'_{2,1})$, since it is a function v_1 from $W^{2m,2}(G_1)$ satisfying the equality $(v_1, A_{2,0}w) = (A'v_1, w)$ for all w in $D(A_{2,0})$.

Remark 3: It is a direct consequence of Theorem 5(a) that if $N(A_2 - \zeta I)$ is trivial, then so is $N(A_p - \zeta I)$ for every p with $1 < p < 2$ (if we apply 5(*a*) to the operator $A - \zeta I$ instead of our original operator A). If G is bounded and we obtain a restriction on ζ for $(A_2 - \zeta I)$ to have a nontrivial null-space which depends only on the regularity constants of (I), (II), and (III), then approximating A by differential operator $A^{(k)}$ uniformly on G, where the $A^{(k)}$ have infinitely differentiable coefficients on the closure of G, we see that each $A_2^{(k)} - \zeta I$ will have a trivial null-space for the excluded region in ζ. By the variational theory of the Dirichlet problem for strongly elliptic operators with regular coefficients, $(A_2{}^{(k)} - \zeta I)^{-1}$ will exist for all sufficiently large k. If the norms of the operators $(A_2{}^{(k)} - \zeta I)^{-1}$ are uniformly bounded for large k (as they will be if inequalities of the form Theorem 1(*a*), 1(*c*), or Theorem 3 have been established), it follows immediately that $(A_2 - \zeta I)^{-1}$ exists on all of $L^2(G)$. For f in $L^p(G)$, however, if $u = (A_2 - \zeta I)^{-1}f$, u lies in $W^{2m,2}(G)$ and for $p^{-1} \geq 2^{-1} - 2m \cdot n^{-1}$, it follows that u and Au both lie in $L^p(G)$. By the sharper form of our *a priori* estimates, u must lie in $W^{2m,p}(G)$. It follows that for such p, $(A_p - \zeta I)^{-1}$ exists. If we continue the boot-strap procedure of which the preceding remarks are the first step, we see finally that $(A_p - \zeta I)^{-1}$ exists for all such ζ for $1 < p < \infty$.

Proof of Theorem 1(a): Under the hypothesis (*I*) that G is uniformly regular of class C^{2m}, given any $\delta > 0$, there exists a family of functions $\{\psi_j\}$ in $C_c^\infty(E^n)$, each having support of diameter less than δ, such that $\sum_j [\psi_j(x)]^2 = 1$ on G, and with the additional property that for any two indices α and β with $|\alpha|, |\beta| \leq 2m$, $\sum_j |D^\alpha \psi_j(x)| \cdot |D^\beta \psi_j(x)| \leq k_{\alpha,\beta}(\delta) < \infty$, where $k_{\alpha,\beta}(\delta) \leq k'_{\alpha,\beta}\delta^{-|\alpha|-|\beta|}$.

Let ξ be a real number, $\xi \geq 0$, u any element of $D(A_2)$. Then, (designating by $\|v\|$ the L^2-norm of v, unless otherwise indicated),

$$\|(A_2 + \xi I)u\|^2 = (Au + \xi u, Au + \xi u) = \|Au\|^2 + \xi^2\|u\|^2 + 2\xi Re(Au, u). \quad (2)$$

By the *a priori* estimate (1) above, $\|Au\|^2 \geq c_0\|u\|^2_{2m,2} - k\|u\|^2$, with constants c_0 and $k > 0$ which are independent of u.

Choose $\delta > 0$, and a corresponding partition of unity $\{\psi_j\}$ as above. We have:

$$(Au, u) = \sum_j \sum_{|\alpha| \leq 2m} (a_\alpha(x) D^\alpha(\psi_j{}^2 u), u). \quad (3)$$

Choosing a point x_j in the support of ψ_j and in G, for $|\alpha| = 2m$,

$$(a_\alpha(x)D^\alpha(\psi_j u), u) = a_\alpha(x_j)\,(D^\alpha(\psi_j u), \psi_j u) + (\{a_\alpha(x) - a_\alpha(x_j)\}D^\alpha(\psi_j u), u) + \sum_{|\beta|,|\lambda|<2m} c_{\beta,\lambda}(a_\alpha(x_j)D^\beta(\psi_j u)D^\lambda u, \psi_j u). \quad (4)$$

Let $w(\delta) = \sum_{|\alpha|=2m} \sup_{|x-y| \leq \delta} \{|a_\alpha(x) - a_\alpha(y)|\}$. Summing (4) over all α with $|\alpha| = 2m$ and combining the result with (3), we obtain,

$$(Au, u) = \sum_j \sum_{|\alpha| \geq 2m} a_\alpha(x_j)\ (D^\alpha(\psi_j u), \psi_j u) + R_1(u) + R_2(u), \tag{5}$$

where

$$\begin{aligned} |R_1(u)| &\leq k_1 \textstyle\sum_{r=0}^{2m-1} \delta^{-2m} \|u\|_{r,2} \|u\| \\ |R_2(u)| &\leq k_2 w(\delta) \|u\|_{2m,2} \|u\|. \end{aligned} \tag{6}$$

By the Fourier transform argument of Van Hove and Gårding, however, $Re\{\sum_j \sum_{|\alpha|=2m} a_\alpha(x_j)\ (D^\alpha(\psi_j u), \psi_j u)\} \geq 0$, for all u in $D(A_2)$ (since all such u satisfy the null Dirichlet boundary conditions in the variational sense). Hence, it follows from (2), (3), (5), and (6) that

$$\begin{aligned} \|(Au) + \xi u)\|^2 \geq c_0 \|u\|^2_{2m,2} - k\|u\|^2 + \xi^2 \|u\|^2 - \\ \xi k_2 w(\delta) \|u\|_{2m,2} \|u\| - \xi k_1 \textstyle\sum_{r=1}^{2m-1} \delta^{-2m} \|u\|_{r,2} \|u\|. \end{aligned} \tag{7}$$

It follows from an elementary analytic argument that there exists a constant k_3 such that for all u in $W^{2m,2}(G)$ on $0 < r < 2m$,

$$\|u\|_{r,2} \leq k_3 \|u\|^{r/2m}_{2m,2} \cdot \|u\|^{(2m-r)/2m}. \tag{8}$$

Using (8), we see that

$$\xi k_1 \delta^{r-2m} \|u\|_{r,2} \|u\| \leq \xi k_1 \delta^{r-2m} \|u\|^{r/2m}_{2m,r} \|u\|^{(4m-r)/2m} \leq$$

$$\frac{c_0}{4 \cdot 2m} \|u\|^2_{2m,2} + \frac{\epsilon}{2} \cdot \frac{\xi^2}{2m} \|u\|^2 + k_4 \delta - 4m \epsilon^{-m/(2m-r)} \|u\|^2. \tag{9}$$

Under the hypotheses of Theorem 5(a), $w(\delta) \to 0$ as $\delta \to 0$. Let $\epsilon > 0$ be given, and choose δ so small that $w(\delta) \leq (c_0 \epsilon)^{1/2} k_2^{-1}$. Then

$$\xi k_2 w(\delta) \|u\|_{2m,2} \|u\| \leq \epsilon \delta \xi^2 \|u\|^2 + k_2^2 [w(\delta)]^2 (4\epsilon)^{-1} \|u\|^2_{2m,2} \leq$$

$$\epsilon^2 \xi \|u\|^2 + \frac{c_0}{4} \|u\|^2_{2m,2}. \tag{10}$$

Summing (9) over r from 0 to $2m - 1$, and combining the result with (7) and (10), we obtain,

$$\|(Au + \xi u)\|^2 \geq \frac{c_0}{2} \|u\|^2_{2m,2} + ((1 - \epsilon)\xi^2 - K(\epsilon, \delta)) \|u\|^2, \tag{11}$$

where $K(\epsilon, \delta)$ is bounded by a rational function of ϵ and δ. In particular, dropping the $(2m)$-norm of u and taking the square root of both sides,

$$\|(Au + \xi u)\| \geq \{(1 - \epsilon)\xi - K_1(\epsilon)\} \|u\|, \tag{12}$$

and 1(a) is proved.

For the proof of Theorem 1(c), we remark that if the coefficients of A of highest order are Hölder-continuous, the function $w(\delta)$ is bounded by a power of δ. In that case, $K_1(\epsilon)$ may be taken in the form $k_5 \epsilon^{-\mu}$ for some positive μ. We have, therefore,

$$\|(Au + \xi u)\| \geq \{(1 - \epsilon)\xi - k_5 \epsilon^{-\mu}\} \|u\|.$$

Choosing $\epsilon = 1/2$ for $\xi \leq 2$, $\epsilon = \xi^{-1}/(\mu + 1)$ for $\xi \geq 2$, we have the inequality of Theorem 1(c) with $\lambda = 1/(r + 1)$.

The further conclusions of (*b*) and (*c*) of Theorem 1 for ζ real follow from Remark

3 and Theorem 5(*a*). The conclusions on the location of the spectrum follow from the first resolvent equation for closed operators.

We shall obtain Theorem 1(*d*) by means of the following result which is of interest in its own right:

THEOREM 6. Suppose that A is an uniformly elliptic differential operator on G, with the boundary Γ of G locally of class C^{4m}, and A and G satisfy the regularity and boundedness conditions (I), (II), (III), and (IV) above. Then:

(*a*) There exist constants $c > 0$, $k \geq 0$, such that for all u in $D(A_2)$,

$$Re(Au, u) \geq c\|u\|^2_{m,2} - k\|u\|^2_{0,2}.$$

(*b*) If A is formally self-adjoint, i.e., $A = A'$, then A_2 is a Hermitian operator in $L^2(G)$, which by (*a*) has its spectrum bounded from below on the real axis.

Proof of Theorem 6: Consider the differential operator $H = {}^1/_2(A + A')$. H is formally self-adjoint, and the hypotheses (I), (II), (III), and (IV) on A imply the corresponding facts for H, which in addition is uniformly elliptic on G since its top-order coefficients are the real parts of the corresponding coefficients of A. Hence by Theorem 5(a) for $p = 2$, if H_2 is the realization of H in $K^2(G)$ under null Dirichlet boundary conditions on the boundary of G (in the sense which we have defined above), then H_2 is a Hermitian operator, i.e., $H_2^* = H_2$. On the other hand, by Theorem 1(*a*) which we have already established, $\|(H_2 + \xi I)u\| \geq \|u\|$ for ξ sufficiently large and positive. Since H_2 is closed, it follows from this latter fact that for such ζ, the range of $(H_2 + \xi I)$ is closed in $L^2(G)$. Since $(H_2 + \xi I)^* = H_2 + \xi I$, the orthogonal complement of this range is the null-space of $H_2 + \xi I$, which by the inequality is trivial. Hence $(H_2 + \xi I)^{-1}$ exists on all of $L^2(G)$ for ζ large and positive, and H_2 must be bounded from below (in the sense of the ordering of Hermitian operators) by $-kI$, i.e. $(H_2u, u) \geq -k(u, u)$ for all u in $D(H_2)$. But $D(H_2) = D(A_2)$, and $Re(A_2u, u) = (H_2u, u)$, which imply that

$$Re(A_2u, u) \geq -k(u, u).$$

Consider finally $A - \epsilon(-1)^m\Delta^m = A_\epsilon$, where ϵ is a small positive constant and Δ is the ordinary Laplace operator in E^n. For ϵ sufficiently small, A_ϵ satisfies all the hypotheses on A and hence for u in $D(A_2) = D(A_{\epsilon,2})$, $Re(A_\epsilon u, u) \geq -k(u, u)$. But, $(A_\epsilon u, u) = (Au, u) - \epsilon(-1)^m(\Delta^m u, u)$, while $(-1)^m(\Delta^m u, u) \geq c\|u\|^2_{m,2}$. Therefore,

$Re(Au, u) = Re(A_\epsilon u, u) + \epsilon(-1)^m(\Delta^m u, u) \geq c\epsilon\|u\|^2_{m,2} - k\|u\|^2$, and all parts of the theorems have been proved.

Proof of Theorem 1(d): We again consider

$$\|(A_2 + \xi I)u\|^2 = (Au, Au) + \xi^2(u, u) + 2\xi Re(Au, u).$$

By theorem 6(a), $Re(Au, u) \geq -k(u, u)$. Hence for $\xi \geq 0$,

$$\|(A_2 + \xi I)\|^2 \geq (\xi^2 - 2k\xi - k_1)\|u\|^2 \geq (\xi - k_6)^2 - k_7^2\|u\|^2.$$

Using the fact that $(a^2 - b^2)^{1/2} \geq a - b$ if $a \geq b \geq 0$, we obtain $\|(A_2 + \xi I)u\| \geq (\xi - k_6 - k_7)\|u\|$. By the same argument, $\|(A_2' + \xi I)u\| \geq (\xi - k_6 - k_7)\|u\|$. The first inequality says that for $\xi > k_6 + k_7$, the range of $A_2 + \xi I$ is closed. Its orthogonal complement is the null-space of $(A_2 + \xi I)' = A_2' + \xi I$, which by the

second inequality is trivial under the same restriction on ξ. Hence, $(A_2 + \xi I)^{-1}$ exists for $\xi > k_8 = k_6 + k_7$, and satisfies the inequality

$$\|(A_2 + \xi I)^{-1}\| \leq (\xi - k_8)^{-1}.$$

The assertion that the spectrum lies in a right half-plane follows once more from the first resolvent equation for closed operators.

Proof of Theorem 3: We proceed as before, except that we now consider $\|(A_2 + \zeta I)u\|$ for $Re(\zeta) \geq 0$, u in $D(A_2)$.

$$\|(A_2 + \zeta I)u\|^2 = \|A_2 u\|^2 + |\zeta|^2\|u\|^2 + 2\,Re\,\{\zeta\,(Au, u)\}.$$

Carrying through the same decomposition as before and using the fact that if A is essentially real, the top-order coefficients a_α are real-valued functions on G, we find

$$Re\,\bar{\xi}(Au, u) \geq Re(\zeta) \sum_j \sum_{|\alpha| = 2m} a_\alpha(x_j)\,(D^\alpha(\psi_j u), \psi_j u) - |\zeta| \cdot \{R_1(u) + R_2(u)\},$$

where $R_1(u)$ and $R_2(u)$ satisfy the inequalities (6) above. If $Re(\zeta) \geq 0$, the first term is nonnegative, and the remainder of the proof is identical with that of Theorem 1(a) if one substitutes $|\zeta|$ for ξ in the latter.

Proof of Theorem 3: We have already observed that $(A_2 - \xi I)^{-1}$ exists for ξ large and negative if G is bounded, and by Remark 3, so does $(A_p - \xi I)^{-1}$. But if $(A_p - \xi I)^{-1}$ exists, it must be compact for G bounded and regular, since for $u = (A_p - \xi I)^{-1}v$, $\|u\|_{2m,\,p} \leq c_p\|v\|$ and on a bounded regular domain sets bounded in $(2m, p)$-norm are pre-compact in $L^p(G)$. The remainder of the assertions follow from the compactness of $(A_p - \xi I)^{-1}$ for any single value of ξ.

Proof of Theorem 4: Let $H = {}^1/_2(A + A')$. H is formally self-adjoint, and if A is essentially real, $A = H + R$, where R is of order $< 2m$. Let R_2 be R restricted to the domain of A_2, H_2 and A_2 as before. Suppose that $R(\zeta) \geq 0$. Then for u in $D(A_2)$,

$$\|(A_2 + \zeta I)u\| \geq \|(H_2 + \zeta I)\| - \|R_2 u\|, \tag{13}$$

$$\|(H_2 + \zeta I)u\|^2 = \|H_2 u\|^2 + |\zeta|^2\|u\|^2 - 2Re(\zeta)\,(H_2 u, u) \geq \{|\zeta|^2 - k_9|\zeta| - k_{10}\}\|u\|^2 + c\|u\|^2_{2m,\,2}. \tag{14}$$

$$\|Ru\| \leq k_{11}\|u\|_{2m-1,\,2} \leq k_{12}\|u\|^{(2m-1)/2}_{2m,\,2}\|u\|^{1/2}. \tag{15}$$

Combining these inequalities, we obtain

$$\|(A_2 + \zeta I)u\| \geq (|\zeta| - k_6|\zeta|^{\lambda_2} - k_6)\|u\|. \tag{16}$$

As before, we see that the range of $(A_2 + \zeta I)$ is closed for $|\zeta|$ sufficiently large in the half-plane $Re(\zeta) \geq 0$. The parallel inequality for $(A_2' + \zeta I)$ implies that the range of $(A_2 + \zeta I)$ is all of $L^2(G)$, while (16) implies for such ζ that $(A_2 + \zeta I)^{-1}$ exists and is a bounded operator. The assertions about the position of the spectrum follow from the inequality (16) and the first resolvent equation.

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[1] These PROCEEDINGS, **45**, 365–372 (1959).

[2] Some of these results (and in particular Theorems 1(d) and 6) were previously announced by the writer in *Comptes Rendus Acad. Sci.*, **246**, 526–528, 1363–1365 (1958).

[3] If $n = 2$, we require the additional condition that for each point x of the boundary, n the unit normal vector to Γ at x, t any unit tangent vector, the polynomial $a(x, t + \lambda n)$ in the complex variable λ should have exactly m roots in the upper-half λ-plane (with multiplicities). This condition is superfluous if A is essentially real.

[4] A sharper form of the *a priori* estimate was given in reference 1 which we apply in the discussion of Theorem 5 and Remark 3, namely that if u lies in $W^{2m,p}$ locally on a neighborhood in G of each point of G u Γ and satisfies the null Dirichlet conditions at each point of Γ, and, in addition, if u and Au lie in $L^p(G)$, then u lies in $D(A_p)$ and (1) holds.

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*MAPPING NORMS**

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1. Introduction.—If X and Y are metric spaces and $f(X) = Y$ is a mapping, we understand by the *norm* $N(f)$ of f the least upper bound of the diameter of point inverses under f, so that

$$N(f) = l.u.b.\{\delta[f^{-1}(y)]\}, \text{ for } y \in Y.$$

This number has entered in various ways into numerous studies of metric and topological properties of mappings and of the spaces on which the mappings act. We shall be concerned with certain cases of the following general question: *For a given metric space X and class T of mappings, under what conditions does there exist a positive constant $d(X, T)$ such that any mapping of the class T operating on X as its domain is necessarily of norm $\geq d(X, T)$?* For example, if X is a circle of radius r and T is the class of nontopological open mappings, it is readily shown that we may take $d(X, T) = r\sqrt{3}$; and, of course, $w = z^3$ is a mapping of this class on the circle $|z| = r$ of norm exactly $r\sqrt{3}$.

Our results have to do largely with the cases in which the spaces X are simple closed curves and the mappings are nontopological open mappings. Application is made to the case where X is an arbitrary 2-dimensional manifold. Proofs for the main theorems are only sketched in this paper and are complete only for the cases of circles, although the statements are made in their full generality. Detailed proofs will follow in a later article.

2. Altitude Norms. If J is a simple closed curve and a, b, and c are any three points on J we denote by $\delta(a, b, c)$ the smallest of the distances $\rho(a, bc)$, $\rho(b, ac)$, $\rho(c, ab)$, where bc is the arc of J not containing a, and so on. We then define the *altitude* $\alpha(J)$ of J as the least upper bound of the aggregate $[\delta(a, b, c)]$ for all triples a, b, c of points on J. It readily follows that actually $\alpha(J)$ is the *maximum* number in this aggregate. Note also that a circle of radius r has altitude $r\sqrt{3}$ in this sense.

2.1. THEOREM. *If J is a simple closed curve, any nontopological open mapping f on J has norm $\geq \alpha(J)$.*

Let $B = f(J)$. The case in which B is a simple closed curve is a direct consequence of the following two statements which we verify.

i. *f is topological on any arc α of J for which*

$$f(\alpha) \neq B.$$

For if α contains two points x_1, x_2 in $f^{-1}(y)$ for some $y \in B$, the subarc x_1x_2 of α contains a component Q of $f^{-1}(J - y)$ and $f(\alpha) \supset \overline{f(Q)} = J - y = J$.

ii. *For any three distinct points a, b, c on J, at least one of the arcs ab, bc, and ca intersects $f^{-1}f(c)$, $f^{-1}f(a)$, or $f^{-1}f(b)$, respectively.*

For if not, each maps topologically onto its image. Also, the arcs $f(ab) = a'b'$ and $f(bc) = b'c'$ can intersect in only $b' = f(b)$, as otherwise either $a'b'$ contains c' or $b'c'$ contains a' contrary to our supposition. Similarly $c'a'$ intersects $b'c'$ in just c' and $a'b'$ in just a'. However, it then follows that f maps C topologically onto J contrary to hypothesis.

Now if B is not a simple closed curve, it must be either a single point or a simple arc. The case of a single point is trivial and that of a simple arc is a consequence of the more general result which now follows.

2.2. THEOREM. *Any mapping of a simple closed curve J into a simple arc (equivalently, any real valued continuous function on J) is of norm $\geq \alpha(J)$.*

For if a, b, and c are points of J mapping into a', b', and c', respectively, either some pair of the points a', b', and c' coincide or else one of them lies between the other two on the arc $f(J)$. Suppose b' is between a' and c'. The $f(ca)$ must contain b' so that $f^{-1}(b')$ intersects ac and we have

$$N(f) \geq \delta[f^{-1}(b')] \geq \rho(b, ac) \geq \delta(a, b, c).$$

This gives $N(f) \geq \alpha(J)$ since a, b, c is an arbitrary point triple on J.

3. Factorizations. The study of mapping norms on simple closed curves may be greatly simplified by some readily verified factorization results which we now develop.

3.1. If $f = f_2f_1$ is any factorization of a mapping $f(X) = Y$, we have

$$N(f) \geq N(f_1).$$

For, let $X' = f_1(X)$ and $x' \in X'$. Then $f^{-1}f_2(x') \supset f_1^{-1}(x')$. This gives

$$\delta[f^{-1}f_2(x')] \geq \delta[f_1^{-1}(x')],$$

and our conclusion follows directly.

3.2. Any open mapping f of even degree $2k$ of a simple closed curve C onto a simple closed curve J admits of factorization

$$f = \phi s$$

where s is a square mapping (topologically equivalent to $w = z^2$ on $|z| = 1$) and ϕ is open and of degree k.

Proof: Let $a \in J$ and let us order the points of $f^{-1}(a)$ cyclically on C: $a_1, a_2, \ldots, a_{2k}$. For each x on the arc $a_1a_2 \ldots a_{k+1}$ of C we define

$$g(a_i) = a_{k+i},$$
$$g(x) = f^{-1}f(x) \cdot a_{k+i}a_{k+i+1}, \text{ for } x \in a_ia_{i+1} - a_i - a_{i+1},$$

where $a_{2k+1} = a_1$. Then, since the open arcs a_ia_{i+1} map topologically onto $J - a$ under f, clearly g is single valued. Now let us decompose C into the point pairs $[x, g(x)]$ for $x \in a_1a_2 \ldots a_{k+1}$, let M be the decomposition space, and $s(C) = M$ the

natural mapping. Then clearly M is a simple closed curve and s is, topologically, a square mapping. Thus, if we define

$$\phi(p) = fs^{-1}(p), \qquad \text{for } x \in M,$$

clearly ϕ is an open mapping of degree k of M onto J and we have

$$f(x) = \phi s(x), \qquad \text{for each } x \in C.$$

NOTE: Since the mapping f is topologically equivalent to the mapping $w = z^{2k}$ on $|z| = 1$, the above result can actually be achieved by using the factorization $w = (z^2)^k$, i.e., $w_0 = z^2$ followed by $w = w_0^k$, of this power mapping.

3.3. Any open mapping f of a simple closed curve C onto a simple arc ab admits of factorization

$$f = \phi r$$

where r is a reflection (topologically equivalent to $w = x + i|y|$ on $|z| = 1$) and ϕ is open.

Proof: Let $a_1 \in f^{-1}(a)$. Proceeding in both directions on C from a_1, let b_1 and b_1' be the first points of $f^{-1}(b)$. Then if $b_1 \neq b_1'$ we proceed from b_1 and from b_1' away from a_1 to the first points a_2 and a_2', respectively, of $f^{-1}(a)$. Again if $a_2 = a_2'$ we proceed from a_2 and a_2' on C away from a_1 to the first points b_2 and b_2', respectively, of $f^{-1}(b)$, and so on. After a finite number of steps (equal to half the degree of f) we reach a point where either $a_n = a_n'$ or $b_n = b_n'$ and the process terminates. The two cases are similar, so we suppose $a_n = a_n'$.

Next we define

$$\begin{aligned} r(x) &= x \text{ for } x \text{ on the arc } a_1b_1a_2b_2 \ldots a_n \text{ of } C \\ r(x) &= f^{-1}f(x) \cdot a_ib_i \text{ for } x \in a_i'b_i' \\ r(x) &= f^{-1}f(x) \cdot b_ia_{i+1} \text{ for } x \in b_i'a_{i+1}' \end{aligned}$$

The clearly $r(x)$ is a retraction of C onto the arc $a_1b_1 \ldots a_n$ and is equivalent, topologically, to a reflection. Further if we define

$$\phi(x) = f(x) \text{ for } x \in a_1b_1 \ldots a_n,$$

clearly ϕ is open and of degree $1/2$ that of f and we have

$$f(x) = \phi r(x) \text{ for all } x \in C.$$

4. Plane Simple Closed Curves. Every simple closed curve in a plane encloses circular disks, of course, and it is possible to relate the size of such disks and the norms of open mappings on the given curve. We have the following general result.

THEOREM. *If the plane simple closed curve C contains or encloses all points of a circular disk of radius r, every nontopological open mapping on C is of norm $\geq r\sqrt{3}$.*

This will be proved here only for the case where C itself is a circle, as the general case presents considerably greater complexity. Actually it is possible to obtain the stronger conclusion that $N(f) \geq 2r$ for any open mapping f on C except in the case where f is of odd degree. In case $f(C)$ is a single point, the result is trivial. Otherwise f is of finite degree k; and if k is even, the image $f(C)$ is either a simple closed curve or a simple arc. We proceed to show (proof valid only for C a circle):

4.1. Any open mapping of even degree on C is of norm $\geq 2r$.

By virtue of the factorization results in §3, we need only consider the cases where f is topologically equivalent either to a square mapping or to a reflection.

Suppose f is (topologically) a square mapping. Let $y \in f(C)$ and let $a + b = f^{-1}(y)$. We suppose C is the circle $|z| = r$ in the complex plane and that a is the point $z = r$. If $b = -r$ we have nothing to prove. Otherwise b is above or below the real axis and we may suppose it is above. Now for any $x \in C$ let us define

$$\theta(x) = \text{amp } x, \text{ so that } 0 \leq \theta(x) < 2\pi.$$

Also for any x on the (upper) arc ab of C, define

$$\begin{aligned} g(x) &= ba.f^{-1}f(x) \text{ for } x \neq a, b \\ g(a) &= b,\ g(b) = a; \text{ and} \\ v(x) &= \theta[g(x)] - \theta(x) \text{ for } x \neq b \\ v(b) &= \lim_{x \to b} v(x) = 2\pi - \theta(b). \end{aligned}$$

Then g and v are continuous and we have

$$\begin{aligned} v(a) &= \theta(b) - \theta(a) = \theta(b) < \pi \\ v(b) &= 2\pi - \theta(b) > \pi. \end{aligned}$$

Accordingly, for some x_1 on ab we have $v(x_1) = \pi$ so that x_1 and $g(x_1)$ are diametrically opposite each other on C. Since $f(x_1) = f[g(x_1)]$, this gives

$$N(f) \geq \rho[x_1, g(x_1)] \geq 2r \text{ (actual equality in the circle case).}$$

The case where f is (topologically) a reflection is handled similarly. For then $f(C)$ is a simple arc $a'b'$ and $f^{-1}(a')$ and $f^{-1}(b')$ are single points a and b, respectively. We may suppose C situated as in the former case with a at $z = r$ and b on the upper semicircle of C. Define $\theta(x)$ and $g(x)$ as before except that this time let $g(a) = a$ and $g(b) = b$. Then we define

$$\begin{aligned} v(x) &= \theta[g(x)] - \theta(x), \text{ for } x \neq a \\ v(a) &= \lim_{x \to a} v(x) = 2\pi. \end{aligned}$$

Again g and v are continuous and we have

$$v(a) = 2\pi,\ v(b) = 0.$$

Thus for some x_1 on ab, $v(x_1) = \pi$ and the conclusion follows as before.

A similar device yields the same stronger conclusion even in certain cases of odd degree as we now show (proof valid only for C a circle).

4.2. If $f(C)$ is a simple closed curve and for some $y \in f(C)$, the set $f^{-1}(y)$ subtends an angle $< \pi$ at the center of the given disk, then $N(f) \geq 2r$.

Again we suppose C situated as in the above proofs and that it coincides with the edge of the given disk. Let the points $a_1, a_2, \ldots, a_k$ of $f^{-1}(y)$ be cyclically ordered on C where k is the degree of f. We may suppose a_1 is the point $z = r$ and that all points of $f^{-1}(y)$ are on the upper semicircle of C. Let $\theta(x)$ be defined as above for all $x \in C$. For $x \in a_1a_2$ we define

$$\begin{aligned} g(x) &= a_ka_1.f^{-1}f(x) \text{ for } x \neq a_1, a_2 \\ g(a_1) &= a_k,\ g(a_2) = a_1 \end{aligned}$$

$$v(x) = \theta[g(x)] - \theta(x) \text{ for } x \neq a_2$$
$$v(a_2) = \lim_{x \to a_2} v(x) = 2\pi - \theta(a_2).$$

Again g and v are continuous and we have

$$v(a_1) = \theta(a_k) - \theta(a_1) = \theta(a_k) < \pi$$
$$v(a_2) = 2\pi - \theta(a_2) > \pi.$$

Thus for some x_1 on a_1a_2, $v(x_1) = \pi$ and our conclusion follows as in the earlier proofs.

Now it is readily seen that the result, 4.2, yields a proof for the theorem stated at the beginning of this section in any case in which $f(C) = J$ is a simple closed curve. For suppose $N(f) < 2r$ and let $y \in J$. Let a and b be points of $f^{-1}(y)$ so chosen that the angle aob at the center o of C which is $< \pi$ is as large as possible. It then follows that this angle aob is $\geq 2\pi/3$. For otherwise it is an immediate consequence of the maximum property of angle aob that the other angle between the rays oa and ob can contain no points whatever of $f^{-1}(y)$. Thus $f^{-1}(y)$ would subtend an angle $< \pi$ at o and we would have $N(f) \geq 2r$.

However, *in case C is a circle* an even better result is readily obtainable as we now show.

4.3. If C is a circle of radius r, any open mapping on C of odd degree $k > 1$ is of norm $\geq r\sqrt{2[1 - \cos(k-1)\pi/k]} = d(r, k)$.

Let f be such a mapping, let $J = f(C)$ and $y \in J$. Then if each of the angles at the center o of C subtended by arcs of C between successive points of $f^{-1}(y)$ is equal to $2\pi/k$, $N(f)$ is seen at once to be exactly equal to the number $d(r, k)$. Otherwise, at least one of these arcs, say ab, subtends an angle $aob < 2\pi/k$.

Now let c be the midpoint of ab and let x_1 be the point of C diametrically opposite c. Then since ab maps onto J, some point x_2 of ab maps onto $f(x_1)$. Then since

$$\sphericalangle x_1ox_2 \geq (k-1)\pi/k,$$

we have

$$N(f) \geq \rho(x_1x_2) \geq r\sqrt{2[1 - \cos(k-1)\pi/k]} = d(r, k).$$

Note that if ϵ is any positive number, we have $d(r, k) > 2r - \epsilon$ for all k sufficiently large. In this form the result can be extended to mappings on arbitrary simple closed curves J. That is, if J is in a plane and contains or encloses all points of a circular disk of radius r, then for any $\epsilon > 0$ all open mappings on J of sufficiently large degree will have norm $\geq 2r - \epsilon$. For an arbitrary J it can be shown that all such mappings of sufficiently high degree have norm $\geq \beta(J) - \epsilon$, where, for any set X,

$$\beta(X) = \underset{a \in X}{g.l.b.} \; [\underset{x \in X}{l.u.b.} \; \rho(a, x)].$$

Note that for a circle C this "rotundity index" $\beta(C)$ is the diameter of C; and for any set X, $\beta(X) \geq {}^1/_2\delta(X)$.

It may be remarked also that the power mappings $w = z^k$ on $|z| = r$ actually attain, for k odd and >1, the mimimum norms $d(r, k)$. Also note that for $k = 3$, $d(r, k)$ reduces to $r\sqrt{3}$, the least possible norm for a nontopological open mapping on a circle of radius r. This mapping of minimum norm is essentially unique as we now show.

4.4. THEOREM. *Let $w = f(z)$ be an open mapping on a circle C of radius r. If $N(f) \leq r\sqrt{3}$, f can be represented in the form*

$$w = f(z) = h(z^3)$$

where h is a homeomorphism.

Thus point inverses are point triples of the form

$$(r, \theta) + \left(r, \theta + \frac{2\pi}{3}\right) + \left(r, \theta + \frac{4\pi}{3}\right), \qquad \text{for } 0 \leq \theta < 2\pi.$$

Proof: Let y be any point whatever of $f(C)$. Then $\delta[(f^{-1}(y)] = r\sqrt{3}$ as otherwise by 4.2 we would have $N(f) = 2r$. Let $a, b \in f^{-1}(y)$ with $\rho(a, b) = r\sqrt{3}$ so that $\angle aob = 2\pi/3$; and let c be the point on C which bisects the larger arc of C from a to b. Now some point on the open arc $bc + ca - b - a$ must belong to $f^{-1}(y)$, as otherwise by 4.2, $N(f) = 2r$. However all points of $bc - b$ are at distance $> r\sqrt{3}$ from a and all points of $ca - a$ at distance $> r\sqrt{3}$ from b. Accordingly the point c and only the point c on this arc $bc + ca - b - a$ belongs to $f^{-1}(y)$. Also no interior point of ab can lie in $f^{-1}(y)$, as all such points are at distance $> r\sqrt{3}$ from c. Thus we have $f^{-1}(y) = a + b + c$; and since y is an arbitrary point of $f(C)$, our theorem follows.

5. Norms for 2-dimensional Manifolds. The above results for simple closed curves, in conjunction with a localization theorem for light open mappings obtained recently by the author,[1] makes possible the proof of existence of a similar type of norm for mappings of this type on arbitrary 2-manifolds. The following theorem can be proved. It represents, in the 2-dimensional case, an extension of the well-known theorem of Newman.[2]

THEOREM. *Let M be any 2-dimensional manifold. There exists a constant $d(M) > 0$ such that any light open mapping on X of degree > 1 at each of an everywhere dense set of image points of M is of norm $\geq d(M)$.*

Indication of Proof: Let A_1 and A_2 be closed 2-cells on M with A_1 lying wholly in the interior of A_2 and so that there is a point $a \in M - A_2$. Let h be a homeomorphism mapping A_2 onto the disk $|z| \leq 2$ in such a way that A_1 maps onto the disk $|z| \leq 1$ under h. Let $\delta > 0$ be determined so that any two points of A_2 at distance apart $\leq \delta$ map into points at distance apart < 1. It then can be shown that $d(M)$ can be taken as the smallest of the three numbers δ, $\rho(A_1, M - A_2)$ and $\rho(a, A_2)$.

This is accomplished by showing with the aid of the Localization Theorem[1] mentioned above that unless the desired relationship holds for a given mapping f, there exists a simple closed curve J in A_2 which encloses A_1 and maps openly under f. Then if we define $g(z) = fh^{-1}(z)$ for z on the disk $|z| \leq 2$, g is light and open on $C = h(J)$; and $N(g) \geq \sqrt{3}$ by the results in §4, since C encloses the disk $|z| \leq 1$. It then follows readily that in any case $N(f) \geq \delta \geq d(M)$.

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SYNTHESIS OF NASCENT PROTEIN BY RIBOSOMES IN ESCHERICHIA COLI

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Introduction.—Cells and tissues of all kinds of living organisms have been found to contain ribonucleic acid (RNA) and protein both as separate constituents and as complexes in the form of ribonucleoprotein particles. From a variety of experimental data it has been inferred that these particles are probably intimately involved in the processes of protein synthesis.

The term microsome was originally applied to the portion of disrupted animal tissue which sedimented between 20,000 and 100,000 $\times$ g.[1, 2] It was found to contain most of the RNA. Later it was established that microsome preparations consisted of an association of ribonucleoprotein particles with fragments of the endoplasmic reticulum and techniques were evolved for removing the latter to yield particles which contained little else besides RNA and protein (see review by Palade[3]). Similar particles have also been isolated from bacteria[4] and the term ribosome has been suggested for the purified particles which are essentially free from lipid and other extraneous matter.[5]

In bacteria, depending on the cultural conditions, up to 80 per cent of the RNA may exist in varying sized ribonucleoprotein particles. These range in sedimentation constants from about 20S to 100S and some preparations have about 2 amino acid residues per ribonucleotide. The distribution among the various classes found in *Escherichia coli* depends on the cultural conditions and the conditions of cell breakage. Exponentially growing organisms possess ribosomes with sedimentation constants approximately 15–20S, 30S, 50S, 70S, 85S, 100S. The last is usually a minor component or absent except in resting cells and the 70S and/or 85S particles predominate over the smaller sizes.[6, 7] The sedimentation constant of the particle which we refer to as 85S does in fact vary between 70S and 100S depending on the magnesium concentration.[8]

Separation of the various particles into homogeneous fractions presents considerable difficulties if attempted by simple differential centrifugation. We have found, however, that centrifugation of cell juices layered on sucrose solutions in the swinging bucket rotor of the Spinco can give a useful resolution of all classes of ribosomes seen in the analytical ultracentrifuge. In addition the isolated fractions are available for analysis. This technique has been used to observe the incorporation of radioactive tracers into the ribosomes and the soluble protein of *E. coli*.

Methods.—The procedures described below were followed except for minor modifications. A radioactive tracer was added to an exponentially growing culture of *E. coli* and, after an appropriate interval, the organisms were fractionated to determine the distribution of the tracer among the various sizes of ribosomes and in the soluble protein.

E. coli B (ATCC No. 11303) was grown overnight at 37°C in an aerated synthetic medium containing glucose (0.4 mg/ml) as carbon source. The yield of organisms was approximately 120μg dry weight per ml of culture and in the morning more

glucose (0.8 mg/ml) was added. This caused immediate resumption of exponential growth. The organisms were harvested after the culture density had about doubled. The cells were washed twice and suspended in a synthetic medium lacking both glucose and the sulfur source. Glucose (0.8 mg/ml) was added and the cells (suspension density of 250 μg/ml) were incubated with aeration at 37°C. Exponential growth was promptly resumed at the expense of endogenous sulfur reserves.[9]

After 30 minutes the culture was poured into a beaker containing carrier-free $Na_2S^{35}O_4$ at a level of 1 mC per liter of culture. After a few seconds the whole suspension was dumped on to half its weight of crushed, frozen medium. This caused the temperature to drop precipitously and effectively halted metabolic activities instantly.

The organisms were then harvested, washed three times with TSM (tris(hydroxymethyl)aminomethane 0.01 *M*, succinic acid 0.004 *M*, MgAcetate 0.01 *M*, pH 7.6)

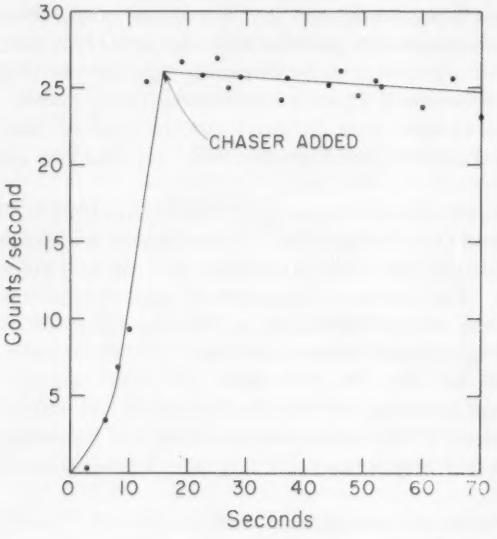

FIG. 1.—Time course of incorporation. $S^{35}O_4^{=}$ was added to a growing culture of sulfur-depleted *E. coli* at time 0. Samples were withdrawn at indicated times, and squirted into TCA, filtered and counted. At 16 seconds $S^{32}O_4^{=}$ S^{32} cystine and methionine were added. Note prompt incorporation of the tracer into TCA precipitable material and rapid cessation of incorporation after addition of nonradioactive material.

and resuspended in TSM (7.5 mg dry weight/ml). The cells were broken either by passing through an orifice under high pressure or by the lysozyme-freezing method.[10] Residual cells, cell walls, and membranes were then removed by centrifugation. The resulting cell juice was then ready for sedimentation analysis as follows.

A mixing device[11] was used to deliver 4.5 ml of a solution of sucrose in TSM linearly graded from 20 per cent w/v to 5% w/v into a centrifuge tube. On this was layered 0.5 ml of a solution graded from 4 per cent to 0 per cent sucrose and containing the cell juice in reversed concentration gradient. This system has inherent stability properties which make it valuable for centrifugal separation of components of different sedimentation constants.[12] The tubes were spun in the swinging bucket head rotor (90,000 g) for periods varying from 75 to 200 minutes. Twenty-five fractions were collected from each tube by puncturing the base with a

hypodermic needle and allowing the contents to drip out into small test tubes. The ultraviolet absorption of the fractions was measured. In addition, the radioactivity precipitable by trichloracetic acid (TCA) was determined. All operations except the final collection of the fractions were carried out at 0–4°C.

Results.—As a preliminary to the study of the incorporation into ribosomes, we investigated the kinetics of $S^{35}O_4^=$ fixation into the total cold TCA precipitable fraction of *E. coli* and the rapidity with which this incorporation could be stopped by lowering the temperature or by adding a "chaser" containing non-radioactive $SO_4^=$, cystine and methionine. Samples were removed with a hypodermic syringe as rapidly as possible from a culture after addition of $S^{35}O_4^=$, squirted into TCA, filtered and counted.[13] After an appropriate time the nonradioactive substrate

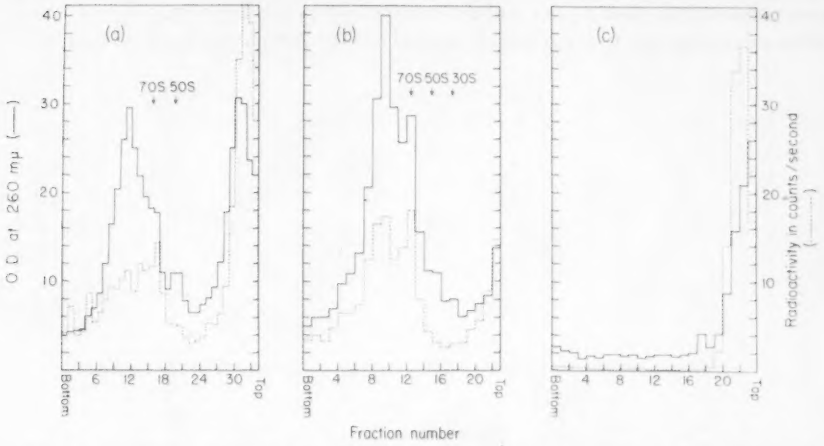

FIG. 2.—Sedimentation analysis of cell juice after 15 seconds incorporation of $S^{35}O_4^=$. $S^{35}O_4^=$ was added to a growing culture of sulfur-depleted *E. coli*. After 15 seconds the culture was chilled, harvested, washed, and broken. The cell walls and membranes were spun out and the juice analyzed in the swinging bucket rotor; 2*a* shows analysis of the total juice; 2*b* analysis of resuspended ribosome pellet; 2*c* analysis of supernatant fluid after removal of ribosomes. The 85S, 70S, 50S, and 30S particles seen in the analytical centrifuge are partially resolved. Note lack of contamination of ribosome region by soluble protein, 2*c*. Centrifugation 75 minutes at 37,000 rpm.

was added and the sampling continued. As shown in Figure 1 a linear rate of incorporation was found after a few seconds and this could be obliterated instantly when the "chaser" was added. These experiments were carried out with cells which had been grown for about 30 minutes without an exogenous source of sulfur. The absence of kinetic delays in the incorporation of S^{35} and in its cessation indicate that under these conditions of sulfur starvation there is little if any pool of precursors to the sulfur amino acids of the cell protein.

In other experiments the culture was poured on ice a few seconds after adding the tracer. Samples taken from the iced suspension during the following 2 hours showed a rate of incorporation less than $^1/_{5000}$ of the rate at 37°. Furthermore, none of the S^{35} which had been previously incorporated into TCA precipitable material was removed by exchange in the presence of chaser at the low temperature.

Accordingly, changes in the content of TCA-precipitable S^{35} occurred during the period when the cells were incubated at 37° and not during the period required for harvesting, washing, and fractionating the cells, all of which were carried out between 0–4°C.

Experiments of a few minutes' duration had consistently failed to show a higher specific radioactivity in the ribosomes than in the soluble protein. We, therefore, investigated the distribution of S^{35} after very short times of incorporation. Organisms were incubated for 10 seconds with $S^{35}O_4^{=}$ and their components were separated by sedimentation analysis. The distribution of TCA-precipitable radioactivity and 260 mμ absorbing material is shown in Figure 2a. Most of the S^{35} is seen to be associated with the ribosomes of the 70–85S class and with the soluble protein which did not sediment under the conditions used. When the ribosomes were pelleted from such a juice and the resuspended pellet similarly analyzed, most of the soluble protein fraction was eliminated but the 70S particles still carried their

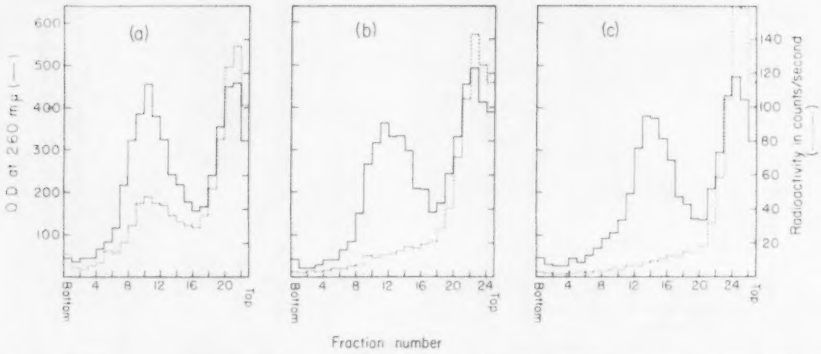

FIG. 3.—Sedimentation analysis of cell juice. (*A*) Cells incubated 15 seconds with $S^{35}O_4^{=}$. (*B*) Cells incubated 15 seconds with $S^{35}O_4^{=}$ followed by 15 seconds incubation with S^{32} "chaser." (*C*) Fifteen seconds incubation with $S^{35}O_4^{=}$ followed by 120 seconds with S^{32} "chaser." Note transfer of radioactivity from 70–85S region to nonsedimenting region. Centrifugation 75 minutes at 37,000 rpm.

original radioactivity (Fig. 2b). This indicates that the S^{35} was relatively firmly bound to the ribosomes (see also below). When the supernatant fluid remaining after the ribosomes had been pelleted was analyzed, very little of the radioactivity moved down the centrifuge tube (Fig. 2c).

In a variety of experiments of short duration (5–20 seconds) the major part of the label associated with ribosomes was found in the 70–85S fraction and the specific radioactivity of these particles was always substantially greater than that of the 30 and 50S ribosomes. When incubations lasting 20, 45, and 120 seconds were used and the resulting cell juices analyzed, it was found that the specific radioactivity of the 70S particles had risen rapidly by 20 seconds and thereafter increased only a little up to 120 seconds. The specific radioactivity of the 50S ribosomes rose more slowly but surpassed that of the larger particles between 45 and 120 seconds. The soluble protein also became labeled rapidly but because of the much greater amount had lower specific radioactivity at the earlier time.

To demonstrate that one component of the cell acts as precursor to another it is necessary to show not only that the radioactivity of the suspected precursor rises rapidly when a tracer substrate is added but also that it is transferred to the product with equal rapidity when the radioactive substrate is replaced by a nonradioactive one. Cells were therefore incubated with the radioactive tracer for 10–15 seconds and then a "chaser" of nonradioactive amino acids was added. After a short period all incorporation was terminated by pouring the culture on ice. Figure 3 shows that most of the radioactivity which was found in the ribosome fraction at the end of 15 seconds was subsequently transferred to the soluble protein during the 15 seconds of incubation with the "chaser." The removal was only slightly greater in 120 seconds. Even a 5 second "chase" was quite effective. Thus there is a protein component which is transiently associated with the ribosomes and has all

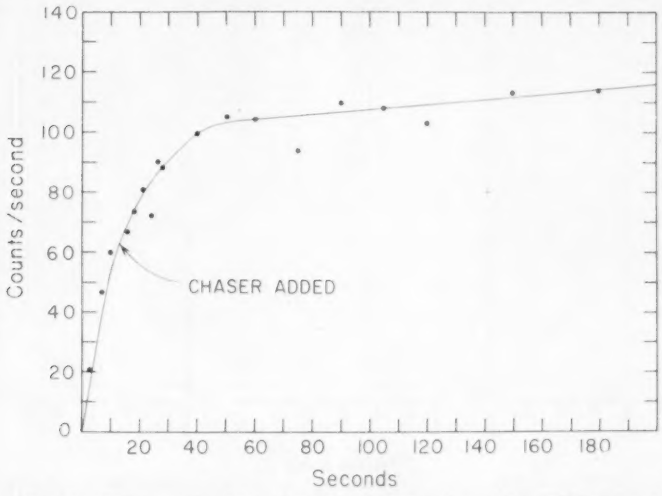

FIG. 4.—Time course of incorporation of C^{14} amino acids. Experimental conditions similar to those of Fig. 1 except C^{14} labeled chlorella protein hydrolysate was used as tracer. Note that incorporation continues after addition of C^{12} amino acids.

the characteristics which would be expected in a compulsory precursor of the soluble proteins. It appears that this nascent protein is a polypeptide strand which is formed on the ribosome and is subsequently released as soluble protein. Owing to the short time periods involved it has not been possible to plot detailed time courses of these processes but three features have been established. (1) The radioactivity of the 70–85S ribosomes built up to a saturation level in 5 seconds or less and died away equally rapidly when the tracer was diluted out. (2) The saturation level was equivalent to the quantity of soluble protein synthesized in three seconds. (3) The decrease in the radioactivity of the ribosomes during the chase was roughly equal to the concomitant increase of radioactivity in the soluble protein.

To check whether the results obtained with S^{35} incorporation were typical of other

amino acids, similar experiments were carried out to observe the incorporation of a mixture of amino acids.† Figure 4 shows that the incorporation of C^{14} from the mixture of C^{14} amino acids (obtained by hydrolyzing *Chlorella* protein) began promptly but could not be terminated rapidly by adding a large excess of C^{12} amino acids. Exchange of exogenous amino acids with the amino acids of the pool is not very rapid and C^{14} continued to enter the protein fraction for roughly 20 seconds after the "chaser" was added. Consequently a longer period was needed to show the transfer of the particle-bound nascent protein to the soluble fraction. The results, however, were quite similar to those obtained with S^{35}. After 5 and 12 seconds a large fraction of the C^{14} incorporated into the TCA-precipitable fraction was associated with the 70–85S particles as shown by the sedimentation analysis. During the subsequent 120 seconds after the addition of C^{12} amino acids the C^{14} was transferred

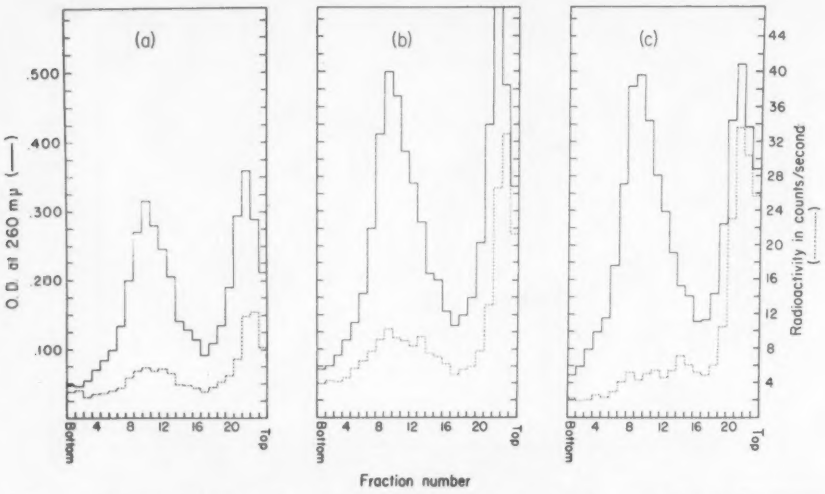

FIG. 5.—Sedimentation analysis of cell juice. (*A*) Cells incubated 5 seconds with C^{14} amino acids. (*B*) Twelve seconds incubation. (*C*) Twelve seconds incubation with C^{14} amino acids followed by 120 seconds incubation with C^{12} amino acids. Note decrease in radioactivity of 70–85S region and increase of radioactivity in nonsedimentable region. Centrifugation 75 minutes at 37,000 rpm.

to the soluble fraction (Fig. 5). As a result of the longer times required to dilute out the free amino acid pools there was more opportunity for incorporation into the structural proteins of the ribosomes and the transfer was not quite so complete as could be observed when using the S^{35} tracer. Neither was it possible to demonstrate the rapidity of the transfer from the particle to the soluble fraction. Nevertheless the results show that the behavior of the S^{35} amino acids is consistent with that of the other amino acids and can be used with confidence to study the processes of protein synthesis.

In the experiments described above we have taken TCA-precipitability as an indication that the labeled amino acids were in peptide linkages. There exist, however, complexes of soluble RNA or lipids with amino acids which are precipitated by cold TCA. A number of tests were therefore carried out to establish the nature

of the labeled material which is transiently associated with the ribosomes. (1) It remained precipitable by TCA after solution in 1 *N* NaOH. (2) It was not extractable by hot alcohol after cold TCA precipitation. (3) It was not exchangeable by incubation with an excess of nonradioactive amino acids. (4) It yielded a large variety of compounds (peptides) having different electrophoretic mobilities after partial hydrolysis by chymotrypsin or 12 *N* HCl. These tests indicate that the bulk of the newly incorporated amino acids which were found associated with the ribosomes have the behavior to be expected of amino acids bound in the peptide linkage.

The association of the nascent protein with the ribosomes, although transient in the growing cell, is quite stable in the disrupted cell juice. Preparations have shown the same specific activity in the ribosome fraction after 5 days at 4°C.

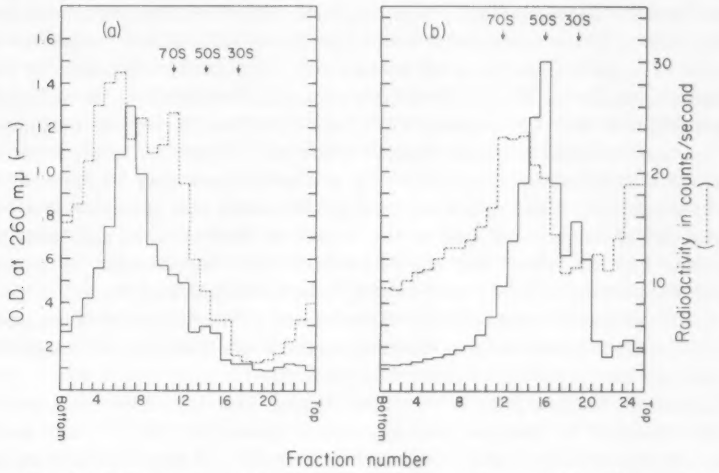

FIG. 6.—Sedimentation analysis of ribosomes from cells exposed for 10 seconds to $S^{35}O_4^{=}$. (*A*) Ribosome pellet resuspended with buffer containing Mg at 10^{-2} M to give mostly 70–85S ribosomes. (*B*) Pellet resuspended with Mg at 10^{-4} M to give mostly 50S and 30S ribosomes.

The ribosomes may be centrifuged down from the cell juice and the pellet shows the same specific radioactivity in the 70S region upon subsequent sedimentation analysis. We have not yet found conditions which cause the release of the nascent protein from the ribosomes. Incubation (15 minutes at 37°) with adenosine triphosphate (ATP) or with amino acids in the presence of cell juice did not release it. Chromatography with diethylaminoethyl (DEAE) cellulose caused disintegration[14] of the ribosomes yielding nucleoprotein which was eluted from the column but leaving about one-half of the ribosome protein and 95 per cent of nascent protein firmly bound. On splitting the ribosomes by decreasing the magnesium concentration of the medium,[15] the nascent protein was found partly in the soluble proteins but mainly in the 50 and 30S particles which comprised the bulk of the nucleoprotein and in a few remaining 70S ribosomes (Fig. 6).

On the other hand, the soluble protein which was labeled with S^{35} as early as 7

seconds after addition of the tracer could not be distinguished from the bulk of the unlabeled soluble protein by chromatography on DEAE. There is no indication from column chromatography that any appreciable time is required (in growing cells) for conversion from nascent protein associated with the ribosomes to the final form of the polypeptide chain.

A less detailed study was made of the incorporation of S^{35} in the fraction of broken cells which sedimented rapidly. This contained fragments of cell walls and membranes as well as some intact cells. Under steady state conditions of labeling roughly 25 per cent of the total S^{35} was in this fraction whereas organisms which had been incubated with tracer for only 10–15 seconds showed about 30 per cent. This might be taken to indicate the presence of a protein precursor. However, sedimentation analysis indicated that half of this material sedimented very rapidly (>1000S) as might be expected for cell walls and unbroken cells. A large part of the remainder is accounted for by contamination with ribosomes and soluble protein leaving only a minor component which has the sedimentation properties to be expected of small fragments of cell membrane. The specific radioactivity of this fraction was similar to that of the soluble protein. Furthermore, its radioactivity did not diminish after the addition of a "chaser." Thus, there is no evidence that this fraction contains a major protein precursor. There is, however, a slight indication that ribosomes associated with cell membranes may be more active in protein synthesis. When cells were lysed by treatment with lysozyme followed by freezing and thawing about half of the ribosomes were released and most of the remainder were detached from the residual membranous material by passing it through the pressure cell. It was found that the first fraction of ribosomes had only about half the specific radioactivity of the second. Possibly some of the particles exist free in the cell juice whereas others are more or less firmly bound to membranes and are more directly involved in protein synthesis.

Discussion.—Various lines of evidence suggest that the microsome system in animal cells may be the predominant site of protein synthesis. For instance, amino acid incorporation studies *in vivo* show that this cell fraction becomes labeled most rapidly and only subsequently does the soluble protein fraction become radioactive.[16,17] Similar results have been obtained with pea seedling preparations.[18] Support for these ideas has come from *in vitro* experiments in which suitably supplemented microsome preparations have been shown to incorporate amino acids into peptide linkage.[19] However, the incorporation was relatively small and boiled preparations were reported to be substantially more active.[20]

In contradistinction, studies with bacterial cell preparations had not hitherto indicated that ribosomes were an important site of protein synthesis. This is surprising in view of the fact that bacteria have a considerably higher content of ribosomes than do animal cells. Some recent reports, in fact, propose that the bacterial cell membrane is a relatively more important structure in such synthesis.[21–23]

In animal tissues, cellular proliferation is small compared with synthesis of soluble protein so there is little difficulty in distinguishing between synthesis of and by ribosomes. In certain cells there is no synthesis of ribonucleoprotein particles so that all incorporation of amino acids can be attributed to synthesis of soluble protein by the ribosomes.[24] In contrast, in growing cultures of bacteria the ribosome content will double in a mean generation time as will the amount of soluble protein.

E. coli in the exponential phase of growth in a synthetic medium has a mean generation time of about 50 minutes and contains approximately 40 per cent soluble protein, 10 per cent ribosomal protein, and 10 per cent cell wall protein, all expressed on a dry weight basis. In a single cell there are roughly 6000 of the 70–85S ribosomes and 6×10^6 arbitrary units of 10,000 MW in the soluble protein. As the growth rate is 0.02%/second, 1.2 ribosomes and 1200 protein units will be synthesized per second. Assuming that the ribosomes are the site of protein synthesis there may be as many as 6,000 sites operating. If this were so, the average time required for the completion of a protein unit would be five seconds. If these nascent protein molecules leave the ribosomes as soon as they are completed, then only those protein molecules synthesized during the previous five seconds will be found adhering to the ribosomes.

At the same time amino acids are flowing in to form the structural proteins of the ribosomes at one-quarter the rate that amino acids are required for synthesis of soluble protein. Hence after twenty seconds the total quantity of new ribosomal protein will equal the quantity of adhering nascent protein. Earlier experiments[14] failed to detect the presence of the nascent protein because periods as long as four minutes were allowed for incorporation. At the end of four minutes the nascent protein would be only 10 per cent of the newly formed ribosome protein.

Fortunately, techniques are available to recognize these two types of synthesis. A 5–15 second "chase" will displace the nascent protein but does not affect the newly formed ribosome protein which is a permanent end product. In other studies carried out in this laboratory[25, 26] it was found that longer incubations (1–30 minutes) with $S^{35}O_4^=$ followed by a short "chase" gave a pattern of labeling which implied that the smaller ribosomes were made first and were themselves precursors to the larger, 30 and 50S particles combining to yield 70 and 85S particles. Accordingly, the two types of synthesis are partially separated according to particle size, the larger particles carrying most if not all of the nascent protein whereas amino acids newly incorporated into the structure of the ribosomes appear at first in the smaller particles. Furthermore, the use of $S^{35}O_4^=$ as a tracer emphasizes the nascent soluble protein as the sulfur amino acid content of the ribosomal protein is one-half to one-third lower than that of the soluble protein. As a result, the radioactivity appearing in the 70–85S particles after incubation with $S^{35}O_4^=$ for 15 seconds or less is chiefly due to nascent protein and there is little contamination from protein of newly formed ribosomes.

The results reported here indicate that a time scale of 5 seconds for the completion of protein units is approximately correct. Free amino acid pools might introduce some slight kinetic delays which would increase the apparent time but there is no doubt that the nascent protein has a transient existence of less than 5–10 seconds in the growing cell.

It is not possible from these experiments to determine the size of the polypeptide strands which make up the nascent protein as this calculation requires a knowledge of the fraction of the ribosomes that is active. The 30 and 50S ribosomes are certainly not the major loci of the nascent protein but they are only minor components and it cannot be excluded that they carry a correspondingly small part of the nascent protein. Their role in forming nascent protein is obscured by the rapid labeling of their structural protein.

It is possible that both the 70 and 85S particles synthesize nascent protein but the former consistently show an initial higher specific radioactivity. Hence, it seems more likely that the nascent protein is formed on the 70S particles and is carried on to the 85S by a rapid interchange between these forms. Finally, it is also possible that only the 70S particles associated with the cell membrane are active. If we assume, however, that the 70S particles are all equally active and that the other particles are inert, and if we take 5 seconds as the time for formation of a polypeptide strand, then the product must have a molecular weight of roughly 20,000.

At first sight it might appear that a growing culture of bacteria is a system so complicated as to be less useful in a study of the problems of protein synthesis than are more simplified systems. It has become apparent, however, that by choosing appropriate time scales one can emphasize one or another aspect of the total cell synthesis. When this is done it is possible to show both the rapid build-up and decay of radioactivity in the nascent protein attached to the ribosomes and to show its transfer to the soluble protein. Furthermore, it is possible to show that this process occurs in the appropriate quantity to account for most if not all of the synthesis of the soluble protein. Finally, it is possible to study separately the synthesis of protein by ribosomes and the synthesis of the ribosomes themselves even though they are intimately coupled in the intact growing cell.

Summary.—The ribosomes of *Escherichia coli* can be separated into different sedimentation classes by centrifugation of cell juices through solutions of sucrose.

Radioactivity from $S^{35}O_4^{=}$ and C^{14}-amino acids rapidly appears in protein bound to the 70S and 85S ribosomes. These become saturated within a few seconds and equally rapidly shed this nascent material as soluble protein which cannot be distinguished from the main bulk of soluble protein.

The rate of formation of this nascent protein on the larger ribosomes is adequate to account for the total cell protein synthesis.

This process is distinct from the concomitant synthesis of the proteins which constitute the permanent structure of the ribosomes.

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† These experiments were carried out with Dr. R. Hendler who used a portion of the cultures to measure the kinetic behavior of lipid-bound amino acids.

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*THE LOWER RANGE OF THE COCHLEAR POTENTIALS**

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Many experiments have been concerned with the functional relation between sound stimuli and the magnitude of the cochlear potentials. Early studies indicated that this function is linear, or nearly so, over a wide range, but departs from linearity when the applied sounds reach extreme intensities. Wever and Bray in 1936, in observations on the guinea pig, obtained curves whose linear range extended from 1 to 150 microvolts at 1,000 cycles per second and from 1 to 100 microvolts at 4,000 cycles, a range of 40 db or more. With the equipment used at that time it was not possible to obtain reliable measurements below 1 microvolt, yet it was pointed out that this value did not represent a lower limit for the response, and doubt was expressed whether any limit exists.

Further studies with improved equipment, and especially with the wave analyzer as a selective voltmeter, extended these measurements a decade lower, to the region of 0.1 microvolts. In this region the function was found to continue its linear form for all tones and in all animal species, including mammals, birds, reptiles, and amphibians. (See, for example, recent studies on cats by Wever, Vernon, Rahm, and Strother, on turtles by Wever and Vernon, and on frogs by Strother.)

Despite this evidence there have been some who have spoken of a "threshold" for the cochlear potentials. Careful consideration shows that in these instances the investigators were misled by the limitations of their measuring equipment, and their "thresholds" merely represented the lowest values that could be read under the conditions of the experiment. The limitations were imposed by background

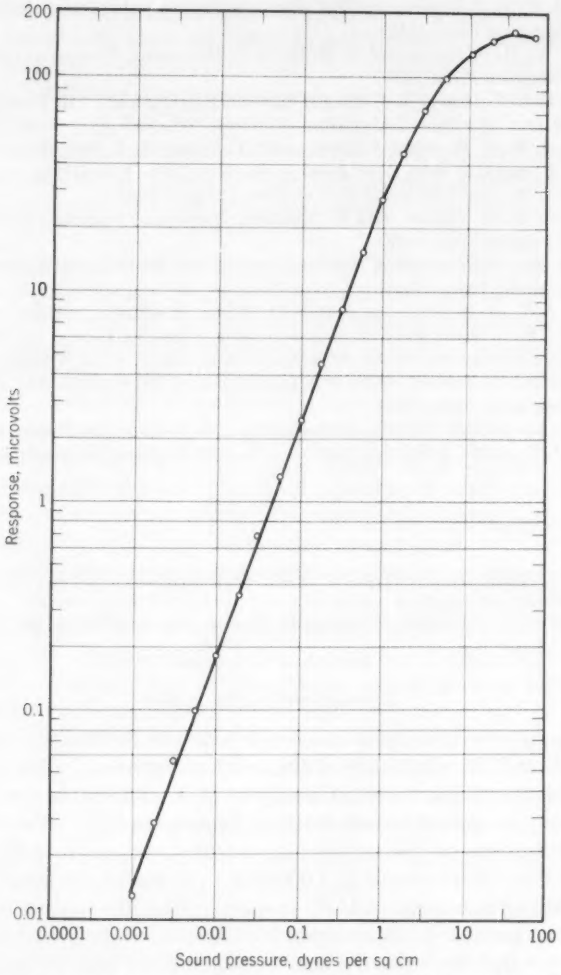

FIG. 1.—An intensity function for the cochlear potentials, recorded from the round window of the cat.

noise arising both in the amplifying apparatus and in the animal itself, and perhaps by other uncontrolled variations.

Our interest in a further exploration of the lower end of the cochlear potential function has been stimulated by general considerations such as those indicated above and also by a recent study by McGill that provides evidence on the actual level of these potentials for sounds at the behavioral threshold. McGill trained cats to respond to a tone by leaping over a barrier to obtain food, and obtained thresholds for eleven tones over a range from 100 to 10,000 cycles. Later the cochlear potentials were measured at the round window on the same animals for these same tones. Functions were obtained over a considerable intensity range,

but it was not possible to make measurements for the identical stimuli that had previously been found to represent the behavioral thresholds. For sounds as faint as these the responses were obscured by background noise. From the measurements made at higher levels, the values representing the behavioral threshold were obtained by extrapolation, on the assumption that the function would continue to be linear at extremely low levels. These extrapolated values varied from 0.034 to 0.0023 microvolts according to frequency. The question of course arises whether this extrapolation is justified.

This question was put to experimental test by the use of a preamplifier of special design giving a gain of 10,000 times and with a particularly low noise level. This amplifier led to a wave analyzer of high selectivity (General Radio 736A). Cats were used as experimental animals, and were deeply anesthetized with diallylbarbituric acid and ethyl carbamate. A platinum foil electrode was placed on the round window membrane by the closed bulla technique, and an indifferent electrode was placed on inactive tissues of the head. A stimulating tone of 5,000 cycles was used because this frequency is well above the greater part of the physiological noise.

Under these conditions, results were obtained as represented in Figure 1. It will be noted that the linear relation now extends from 0.01 microvolts to 50 microvolts or a little above, a range of 75 db. The lowest measurements now extend into the region of the behavioral thresholds as determined by McGill, and yet without indication that the potentials themselves have approached a limit. Our expectation is that a further refinement of the methods of measurement would extend the linearity still farther downward.

Our conclusion is that the cochlear potentials bear a linear relation to sound pressure from the region of the behavioral threshold, or perhaps below it, all the way to the level at which the ear begins to overload. Evidently the process by which sounds give rise to the cochlear potential is a simple and direct one, such as a change in the form of the sensory cells, and this change is proportional to the sound pressure from zero upward to the region of overloading. A threshold appears only in the arousal of nerve impulses by this sensory activity.

For most experimental purposes it is convenient to make measurements somewhere in the middle of the linear range, where there is no disturbance from background noise. From such measurements an extrapolation downward is fully justified.

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